ABSTRACT

HOLANDA, DEBORA MURATORI. Nutritional Strategies to Manage Challenges of Multiple Mycotoxins in Feeds for Nursery Pigs. (Under the direction of Dr. Sung Woo Kim).

Aflatoxins and *Fusarium* toxins are the most prevalent mycotoxins and are responsible for major losses in the pig industry. For instance, such losses are estimates in $ 1.4 billion a year in the United States considering agriculture and livestock impacts. Mycotoxins of great interest are aflatoxins, deoxynivalenol, and fumonisins, because of their elevated global frequency and high occurrence in corn, which is the main feedstuff in pig diets. The toxic effects of aflatoxins, deoxynivalenol, and fumonisins are more pronounced in young pigs and include immune modulation, intestinal barrier disruption, and cell toxicity, resulting in impaired pig performance. Mycotoxin mitigators are a current need to enable sustainable use of feedstuffs and feeds while reducing the detrimental effects of mycotoxins. Dietary additives to mitigate mycotoxicosis often combine organic and inorganic sources to enhance individual adsorbability, immune stimulation, or ability to render mycotoxins less toxic. An emphasis is given to deoxynivalenol because of its frequency (up to 95%) and the lack of an efficient mitigator. Besides, a current trend in swine production is the use of non-artificial feed additives. Thus, the use of yeast-based products is preferred to chemicals or industrialized products as mycotoxin mitigators.

In the first study the effects of mycotoxins, of a yeast-based additive (PYCW; Nicholasville, KY, USA), and of PYCW in mitigating mycotoxin toxic effects in newly-weaned pigs were investigated. Forty-eight newly-weaned pigs (21-d-old) were individually assigned to four dietary treatments following a 2 x 2 factorial arrangement. Factors were: dietary mycotoxins (deoxynivalenol: supplemental 2000 µg/kg in 3 phases; and aflatoxin: supplemental 200 µg/kg only in phase 3) and PYCW (0.2%). The outcomes showed that mycotoxins impaired growth

performance and nutrient digestibility in newly-weaned pigs, whereas PYCW could partially improve pig health regardless of mycotoxin challenge.

The second study investigated the efficacy of yeast-based mycotoxin mitigators on the health and growth performance of newly-weaned pigs (27-d-old) challenged with deoxynivalenol. Sixty pigs were individually assigned to five treatments: NC (negative control, 1.2 mg/kg of deoxynivalenol); PC (positive control, 3.2 mg/kg of deoxynivalenol); CYC (PC + clay/yeast culture-based product, 0.2%); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants-based product, 0.2%); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants-based product, 0.2%). Deoxynivalenol increased the oxidative stress but moderately impaired the health and growth performance of pigs. The mycotoxin mitigators could partially overcome deoxynivalenol toxicity by reducing oxidative stress and enhancing immune function and gut health.

The third study investigated the effects of a yeast and enzyme-based mycotoxin mitigator (MegaFix®, ICC, São Paulo, Brazil) on the health and growth of newly-weaned pigs challenged with deoxynivalenol. Thirty-six pigs (27-d-old) were individually assigned to 3 treatments: CON (minimal deoxynivalenol), MT (supplemental 1.9 mg/kg of deoxynivalenol), and MT+D (MT + mycotoxin mitigator, 0.2%). Deoxynivalenol did not impact growth but increased oxidative stress and partially debilitated the hepatic health of pigs. The mycotoxin mitigator enhanced hepatic health and attenuated gut damage in pigs fed deoxynivalenol.

The fourth study investigated the effects of mycotoxin challenge on the growth and health of pigs with different weaning weights. Out of 106 weanling pigs, 10 were sampled on d 0. Ninety-six pigs were allotted to four treatments following a 2 x 2 factorial arrangement with 3 pigs per pen. Factors were: weaning weight, light (< 7.5 kg) or heavy (> 9.0 kg); and dietary

mycotoxin challenge, low or high (supplemental 0.2 mg/kg aflatoxins, 2.0 mg/kg deoxynivalenol). Light pigs had a more immature microbiome, higher intestinal immune activation, inflammation, and soft feces due to weaning stress. The higher intestinal immune activation, oxidative stress, and incidence of soft feces persisted in light pigs regardless of mycotoxin challenge. Mycotoxins caused intestinal inflammation and impaired growth performance regardless of weaning weight.

In conclusion, yeast-based mycotoxin mitigators partially overcame the detrimental effects caused by mycotoxins on the health and growth of newly-weaned pigs. Light pigs have a less developed microbiome and are more susceptible to weaning stress impacts on gut health and growth. Light and heavy pigs were equivalently susceptible to mycotoxins. Although, more investigation is necessary to find an efficient mycotoxin mitigator to overcoming the toxic effects of mycotoxins, particularly deoxynivalenol.

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Nutritional Strategies to Manage Challenges of Multiple Mycotoxins in Feeds for Nursery Pigs

by
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BIOGRAPHY

Débora Muratori Holanda is from Muriaé, a small city located in Brazil. At a younger age, she already showed interest in the family farm and animal production. This affection moved her to pursue a Veterinary Medicine career at the Universidade Federal de Viçosa (UFV), where she earned her Bachelor's degree. After her graduation, she worked for a small period at a pig farm, where she developed an interest in more scientific knowledge about the matter. Therefore, she started her Master's at UFV in 2015, focusing on pig nutrition, followed by a doctoral student career at North Carolina State University (NCSU) centering in nursery pig nutrition and mycotoxins. Working in one of the greatest animal science research institutions improved her learning, scientific skills, and ability to deal and solve problems in animal production. She intends to continue her professional career working with pig nutrition and research.

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CHAPTER 1. LITERATURE REVIEW

1.1. Abstract

This review aimed to investigate the occurrence of mycotoxins, their toxic effects, and dietary mitigation additives discussed in scientific publications related to pig production. Mycotoxins of major interest are aflatoxins and *Fusarium* toxins, such as deoxynivalenol and fumonisins, because of their elevated frequency at a global scale and high occurrence in corn, which is the main feedstuff in pig diets. The toxic effects of aflatoxins, deoxynivalenol, and fumonisins include immune modulation, disruption of intestinal barrier function, and cell toxicity leading to cell death, which all result in impaired pig performance. Dietary additives currently available to mitigate mycotoxicosis often combine organic and inorganic sources to enhance individual adsorbability, immune stimulation, or ability to render mycotoxins less toxic. In summary, mycotoxins are a challenge to pig production worldwide because of their increasing occurrence in recent years and their toxic effects impairing the health and growth of pigs. Effective dietary mitigation additives must be used to boost pig health and performance, and to improve the sustainable use of crops.

Keywords: detoxification; mycotoxin; occurrence; pig; toxicity

1.2. Introduction

Mycotoxins are secondary metabolites naturally produced by fungi that may have toxic effects. For instance, mycotoxins may present negative effects when fed to livestock animals in contaminated feedstuffs. The mycotoxin contamination in feedstuffs can occur in farms, post-harvesting, or during storage [1]. *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* are among the most frequent genera of fungi to cause intoxications [1,2]. Studies have shown that feedstuffs and finished feeds are found to be contaminated with mycotoxins frequently and ubiquitously. More than 70% of animal feeds and feedstuffs produced worldwide are contaminated with at least one mycotoxin, where the most prevalent mycotoxins are deoxynivalenol, aflatoxin B1, and fumonisins [1,3,4].

In pig production, mycotoxins are known to impair the health and growth of animals. Due to the toxic effects of mycotoxins, the Food and Drug Administration set advisory levels for mycotoxins in the United States. For nursery pigs, mycotoxin concentration must not surpass 0.02 mg/kg for aflatoxins, 1 mg/kg for deoxynivalenol, and 10 mg/kg for fumonisins in the finished feeds [5]. Similarly, the European Union stipulated 0.01, 0.9, 5 mg/kg for aflatoxin B1, deoxynivalenol, and fumonisins, respectively [6,7]. The losses derived from poor animal performance caused by mycotoxins are not alone. The economic impact of the three most frequent mycotoxins (aflatoxins, deoxynivalenol, and fumonisins) considering agriculture, livestock, and mitigation strategies (without considering the direct impact on human health) was estimated at $1.4 billion annually in the United States [8]. This yet concerning mycotoxin scenario is expected to be aggravated by global warming with a higher prevalence or levels of mycotoxin contamination [9–11].

Therefore, investigations for feed additives to mitigate the toxic effects of mycotoxins are increasing. The use of dietary mitigation additives is advantageous for reducing the toxic effects of mycotoxins in pigs and, at the same time, may reduce the waste of crops and enable more sustainable use of feedstuffs. There are many mechanisms by which feed additives mitigate the toxic effects of mycotoxins in feed. One of such mechanisms is by adsorption, where the mycotoxin interacts with another molecule (adsorbent) and is trapped from being absorbed from the gastrointestinal lumen. This way, the mycotoxin will be excreted in the feces and its toxic effects will be minimized in the organism. Another mechanism is to use additives to boost immune function and gut health, turning the animal less susceptible to the toxic effects of mycotoxins. These additives include the use of prebiotics and probiotics, essential oils, algal extracts, plant derivatives, antioxidants, and others [12].

For this review, aflatoxins, deoxynivalenol, and fumonisins were selected to be covered based on the impact of such mycotoxins in pig production (mycotoxin frequency, their toxic effects in the pig, and the existence of official regulations). In addition, this review covered dietary mitigation additives, which are likely to become more frequently employed because of the growing mycotoxin contamination and the need to optimize the utilization of food and feed products.

1.3. Mycotoxin occurrence

The initial mycotoxin contamination can occur at the crop farm (before or during harvesting) or during storage, transportation, feed manufacturing, or at the animal farm prior to pig consumption [1]. Mycotoxin contamination may also be influenced by the type of feedstuff, thus affecting the final contamination in pig feed. Therefore, it is important to measure the

incidence and the concentration of mycotoxins in feedstuffs and feeds before pigs access them. Besides the initial contamination, other factors may predispose to fungal development and, eventually, increase mycotoxin contamination. For instance, the occurrence of mycotoxins may differ depending on the location, but this variation is likely to be influenced by the weather conditions. Global mycotoxin occurrence and concentration in feedstuffs and feeds used for pigs as well as influencing factors on mycotoxin occurrence, testing, and results will be discussed in this section.

Mycotoxin contamination in feedstuffs and feeds is observed globally. Recent publications regarding the occurrence of mycotoxins are summarized in Table 1. In a ten-year-period, it was observed that 88% of samples were contaminated with at least one mycotoxin in an investigation involving 100 countries [4]. Similar outcomes were observed in an eight-year-study comprising 82 countries, where 72% of samples were contaminated with mycotoxins [3]. Specifically in pig feeds, 96% of samples were found to be contaminated with at least one mycotoxin globally [13].

Overall, the *Fusarium* toxins were the most frequently detected. Either fumonisins, deoxynivalenol, or zearalenone ranked first as most detected mycotoxins across studies [3,4]. Similar behavior was observed when geographic regions were considered. Even in studies with a broad survey, including assessment of emerging mycotoxins, which are frequently overlooked, zearalenone ranked at first place in occurrence [13]. Exceptions would be in Sub-Saharan Africa and South Asia, where aflatoxin B1 ranked first [4].

Table 1. Frequency and occurrence of single or multiple mycotoxins according to sample type and origin.

Period of sampling	Samples	Origin	Top 4 mycotoxins detected (frequency)	Samples positive to		Reference
				Single mycotoxin	Multiple mycotoxins	
2004 to 2011	17,316 - feed and feedstuff	Global	DON (55%), FUM (54%), ZEA (36%), AFL (27%)	72%	38%	[3]
2008 to 2019	74,821 - feed and feedstuff	Global	DON (64%), FUM (60%), ZEA (45%), AFB1 (23%)	88%	64%	[4]
2016	595	United States	Type B trichothecenes (85%), FUM (61%), ZEA (51%), AFL (5%)	-	≥ 85%	[14]
2017	733	United States	Type B trichothecenes (78%), FUM (43%), ZEA (32%), AFL (1%)	-	≥ 78%	[14]
2018	147 - corn and corn derivatives	United states	Type B trichothecenes (56%), FUM (64%), ZEA (31%), AFL (10%)	-	≥ 56%	[14]
2011 to 2014	1,384 - corn, corn silage, cereals, feed	Poland	DON (95%), NIV (85%), T-2 (79%), HT-2 (85%)	68%	-	[15]
2014 to 2018	524 - finished feed for pigs	Global	ZEA (96%), brevianamide F (95%), culmorin (94%), maculosin (94%)	≥ 96%	88%	[13]
2010 to 2012		Europe (85.5%), America (9.6%), Australia (4.8%)	Beauvericin (98%), ennitatins (96%), DON (89%), emodin (89%)	100%	100%	[16]

DON, deoxynivalenol; FUM, fumonisins; ZEA, zearalenone; AFL, aflatoxins; AFB1, aflatoxin B1; T2, T2 toxin; and HT2, hydroxy-T2 toxin. The dash "-" is used when the information could not be retrieved from the publication.

Corn is a major feedstuff used globally for feeding pigs, turning its investigation for mycotoxin contamination valuable to trace and estimate mycotoxin occurrence related to pig

production. Among corn samples, the most frequent mycotoxins were fumonisins, followed by deoxynivalenol, and zearalenone [3]. Alarmingly, according to Streit et al. [3], corn samples presented the highest occurrence (84%) and levels of mycotoxins than the average across samples tested, except for ochratoxin A. As expected, the same study found similar contamination levels between finished feed and corn, as corn is the main component in swine feed formulation. Comparable outcomes were observed by other researchers, where both in corn and finished feed the most frequently detected mycotoxins were fumonisins, deoxynivalenol, and zearalenone [4]. The same authors reported that finished feed samples showed higher occurrence for most mycotoxins tested, as expected, because of the combination of a variety of feedstuffs into the finished feed.

The effect of weather conditions on mycotoxin occurrence was observed in a trend of increased incidence across different mycotoxins in South-East Asia, as severe rainy and dry seasons were observed in the same period [3]. Aflatoxin B1, deoxynivalenol, and fumonisins contamination were similarly related to weather conditions favoring crop contamination [4]. Confirming adverse weather as a contributing factor to mycotoxin occurrence, it is expected higher mycotoxin occurrence and contamination levels in the future due to global warming [17,18]. Under global warming, crops harvested out of the tropical area are expected to become more susceptible to fungal diseases and, thus, mycotoxin contamination [11]. Nevertheless, collected crop samples are not obligatorily harvested, stored, and processed in the same place (country), thus, this can add additional confounding factors to obtained results if data are not attentively recorded.

Another factor that may influence the outcome observed in scientific studies is the number of mycotoxins tested and how data are presented. In a study assessing 320 fungal

secondary metabolites, it was observed that the concentration of mycotoxins can be highly variable depending on whether the median or the average concentration is reported [16]. Such variation happened due to the occurrence of a few samples with exceptionally increased concentrations pushing the average upwards. Thus, reporting average concentrations may not truly represent the level of mycotoxin contamination in samples and the median may be more representative for the description of a data set. Similar outcomes regarding average and median concentrations were also observed in a global survey of mycotoxin occurrence by Marin et al. [19]. To account for this variability, Gruber-Dorninger et al. [4] assessed the percentage of samples that surpassed the advisory levels of mycotoxin by the European Union considering 14 geographic regions. The percentage of samples that surpassed the advisory levels for all regions on average were respectively 15.44, 2.29, 10.61, 10.42, and 0.79% for aflatoxin B1, fumonisins, zearalenone, deoxynivalenol, ochratoxin A. These data show that 10% or more of samples are contaminated with aflatoxins, zearalenone, or deoxynivalenol at levels that may have detrimental effects on animal production. However, the data presented by the authors does not allow estimating co-contamination with mycotoxins. Thus, it is likely that the percentage of samples contaminated with any given mycotoxin will be higher than considering individual mycotoxins when one exceeds the preconized levels by the European Union.

Also, the variety and types of mycotoxins tested may influence the outcomes. Mycotoxin testing on samples changes across studies and within the same study. Streit et al. [3] reported that the majority of wheat samples tested in eight years were tested for deoxynivalenol and zearalenone only. The lack of uniformity on sample testing may bias the outcomes of the studies. In addition, there are metabolites from fungal metabolism which are frequently overlooked in mycotoxin analyses. These metabolites are commonly known as “emerging” mycotoxins, which

are currently unregulated and, thus, not considered in most of the tests [20]. The second class of commonly neglected mycotoxins is the “masked” or “modified” mycotoxins. The modified mycotoxins are those that underwent modification in their chemical structure and, thus, are not detected in conventional mycotoxin tests [21]. Even though most of the current reports regarding mycotoxin occurrence lack a screening including emerging and modified mycotoxins, current data are informative and may set guidelines for future investigations.

In the following sub-sections, the occurrence of major mycotoxins (aflatoxins, deoxynivalenol, and fumonisins) are detailed individually for a better understanding of their occurrence and its relation with the stipulated advisory levels in the United States and the European Union. Also, the concomitant occurrence of mycotoxins is discussed in the last sub-section because of its high incidence and increased likelihood of happening in the current pig production scenario.

1.3.1. Aflatoxins

Aflatoxins are produced by fungi of the *Aspergillus* genus. The *Aspergillus flavus* commonly contaminates grains and nuts with aflatoxins during the preharvest period [22]. The *A. flavus* is known to produce aflatoxins B1 and B2. Another species, *Aspergillus parasiticus* can produce aflatoxins G1 and G2 in addition to the aflatoxins produced by *A. flavus* [22].

To limit the toxic effects of aflatoxins, the Food and Drug Administration sets a guidance level of 0.2, 0.1, and 0.02 mg/kg for pigs over 100 lb (about 45.5 kg), breeding animals, and immature animals (less than 4 months of age), respectively, for the sum of aflatoxins in the United States [23]. The European Commission has advisory limits for aflatoxin B1 contamination in feedstuffs and feeds for young pigs at 0.01 mg/kg and 0.02 mg/kg for older pigs [6]. The regulation of solely aflatoxin B1 is due to its greater toxicity as well as higher

occurrence and concentration over other aflatoxins as a contaminant in feedstuffs and finished feeds. Therefore, only aflatoxin B1 is regulated in the European Union as indicative of contamination by other aflatoxins as well. Reinforcing such regulation, in a study where most of the samples were European and from finished feeds, a small percentage of samples tested positive for aflatoxins (2%) but all of those samples were positive for aflatoxin B1 [16]. Additionally, aflatoxin B1 was detected as the most frequent mycotoxin among non-*Fusarium* toxins [3,4,14], showing the importance of setting guidance levels for such frequent mycotoxin. Specifically for corn, contamination with aflatoxin B1 was correlated with increased temperatures and precipitation close to silking and harvesting periods of corn [4].

1.3.2. Deoxynivalenol

Deoxynivalenol is a type B trichothecene, a naturally occurring metabolite of fungi from *Fusarium* genus which may infect feedstuffs used in feed formulation. For instance, *F. graminearum* and *F. culmorum* are the main species producers of deoxynivalenol globally [24]. *Fusarium* toxins were the most frequent mycotoxins globally in the past ten years [4]. Deoxynivalenol was the most frequent, with more than two-thirds of samples of feedstuffs positive for it [4]. Among feedstuffs positive for deoxynivalenol, corn and wheat were the most frequently contaminated [4,14]. Contamination of crops with deoxynivalenol was correlated with mild temperatures and increased precipitation during flowering and maturation periods [4]. Due to the high occurrence and deleterious effects of deoxynivalenol, there are governmental regulations in the United States and the European Union. The Food and Drug Administration has advisory levels for not surpassing 1 mg/kg of deoxynivalenol in feeds for pigs [5], whereas the European Commission stipulated 0.9 mg/kg of deoxynivalenol in feeds for pigs [7]. A concerning outcome was observed in corn sampled over a three-year period in the United States.

Type B trichothecenes occurred in 78% of samples with an average concentration of 1.2 mg/kg [14]. In European, Asian, and Pacific countries, *Fusarium* toxins are the most frequent, with the type B trichothecene, deoxynivalenol, ranking at first [25]. In finished pig feed, similar results were observed where deoxynivalenol was detected in 88% of samples [13]. Although deoxynivalenol concentration in contaminated samples can have a wide range (0-50 mg/kg), most samples are below 5 mg/kg [26]. However, 5 mg/kg is 5-fold higher than official guidelines in many countries.

1.3.3. Fumonisins

Fumonisins are also *Fusarium* toxins, being mainly found worldwide in crops contaminated with *F. verticilliodes* and *F. proliferatum* or locally by *F. nygamai*, *F. napiforme*, and *F. globosum* [24]. Contamination of crops with fumonisins was correlated with increased temperatures and decreased rainfall during silking [4]. Fumonisins are mainly found as contaminants in corn and, as a consequence, in finished feeds [3]. The advisory levels set for fumonisins comprises the sum of fumonisins B1 and B2 at 5 mg/kg of finished feed for pigs in the European Union [7], and the sum of fumonisins B1, B2, and B3 at 10 mg/kg of finished feed in the United States [27]. Of interest, fumonisins ranked in order of occurrence are: B1, B2, B3, and B4 [16]. Thus, justifying the use of the two (European Union) or the three (United States) most frequent mycotoxins among fumonisins as indicative of their overall contamination.

1.3.4. Co-occurrence

Even though most of the samples are under the limits and guidance levels set by the European and United States authorities, a considerable amount (38-64%) of samples are contaminated with more than a single mycotoxin [3,4]. This high incidence of co-contamination

shows the need to investigate the association and the interaction of the effects of co-occurring mycotoxins in pigs. For instance, diets naturally contaminated with deoxynivalenol may impair pig growth at 0.6 mg/kg of diet, whereas for diets artificially contaminated, with purified deoxynivalenol, growth impairment is observed at 1.8 mg/kg of diet [26].

Not surprisingly, and similar to the results observed for single mycotoxin occurrence, the mycotoxins most frequently found as co-contaminants for corn and finished feed were fumonisins, deoxynivalenol, and zearalenone for both global and regional assessments [4]. Among corn samples, 46% of the samples were co-contaminated [3]. A study that analyzed 524 samples of finished feeds for pigs found that 88% of the samples were contaminated with deoxynivalenol and, in addition, all deoxynivalenol-positive samples had a co-contaminating mycotoxin [13]. Out of the co-contaminants detected, 9 mycotoxins were found in 90% or more of the samples along with deoxynivalenol (culmorin, 99%; zearalenone, 96%; brevianamide F, 95%; maculosin, 94%; enniatin B1, 92%; enniatin B, 91%; asperglaucide, 90%; emodin, 90%; and moniliformin, 90%) and, of note, 8 of these are considered as emerging mycotoxins. A concerning scenario was observed by Streit et al. [16] where all samples collected, mostly in Europe, were contaminated with mycotoxins. Yet more alarmingly, all samples had at least 7 and at most 69 co-contaminants detected.

Overall, mycotoxins are found to be contaminants in several feedstuffs as well as in finished feeds and they occur ubiquitously. The majority of the samples tested positive are contaminated with multiple mycotoxins. With a few exceptions, *Fusarium* toxins are the most frequently mycotoxins detected regardless of sample types and geographic regions. Among all mycotoxins, deoxynivalenol, aflatoxins, and zearalenone are more frequently observed above the levels that may cause toxic effects in animals. Therefore, understanding the occurrence as well as

the toxic effects of mycotoxins in pigs work together on finding the best choice of mitigation approach to be used in pig production.

1.4. Mycotoxin toxicity

Mycotoxins, when ingested by pigs, can cause toxic effects impairing the health and growth of pigs. Even though zearalenone is among the most frequently detected mycotoxins, this review will further discuss aflatoxins, deoxynivalenol, and fumonisins because of the controversial effect of zearalenone in pig growth performance and the absence of regulation for this mycotoxin in the United States, China, Brazil, and other key pig-producing countries.

1.4.1. Aflatoxins

Aflatoxins inhibit RNA polymerase transcription of DNA to mRNA in the nucleus, reducing cell protein synthesis [28], thus, increasing cell toxicity and death [29]. Aflatoxin B1 shows higher toxicity and carcinogenic effects in comparison to other aflatoxins [22]. Aflatoxin B1 may suppress antigen-presenting cells by altering the function of dendritic cells and eventually reducing T-cell proliferation and differentiation [30]. Under chronic exposure, aflatoxin B1 can lead to immune suppression, hepatic damage, impaired growth, and may interact with the DNA leading to neoplasia development [31,32].

Indeed, aflatoxin B1 has caused detrimental effects on liver health and electrolyte balance in pigs [33]. Aflatoxins also cause impaired animal growth due to cytokine release [34]. Mycotoxins lead to impaired function and altered architecture of the liver and kidney [35,36]. The effects of mycotoxins in these two organs with important metabolic functions may influence cholesterol synthesis and, later, vitamin D activation as well as calcium and phosphorus balance

[37,38]. Supporting the effect of mycotoxins on vitamin D metabolism, it was previously demonstrated the toxic effects of aflatoxins on the kidney, and vitamin D and calcium levels in poultry [39]. In case of ingestion of aflatoxins, the liver has a central role in the detoxification. The cytochrome P450 can either convert aflatoxins to its epoxide and more toxic form or to aflatoxins M1 and M2, which are less toxic [22].

1.4.2. Deoxynivalenol

The dietary intake of deoxynivalenol is known to reduce feed intake and gain of pigs [40]. Pigs start showing reduced growth performance when fed from 1 to 3 mg/kg of deoxynivalenol [19]. Specifically for naturally contaminated diets, concentrations of 1-2 mg/kg of deoxynivalenol reduce feed intake and gain where each additional 1 mg/kg of deoxynivalenol further reduces gain by 8% in pigs [26].

Deoxynivalenol reduces feed intake in animals, especially in pigs, by modulating local serotonin and decreasing bowel movements [41,42], and increasing satiety signaling [43], the release of pro-inflammatory cytokines [44], and may cause vomiting [45]. In mice, deoxynivalenol reduced feed intake in 2 hours after mycotoxin administration and at a dose-dependent response [43]. In addition to reduced feed intake, growth is diminished by deoxynivalenol-induced disruption of the intestinal barrier and increased intestinal permeability by activation of the mitogen-activated protein kinase pathway in pigs [46]. At high concentrations, it was shown that deoxynivalenol at 10 mg/kg in feed can reduce the digestibility of essential amino acids in pigs [47]. Deoxynivalenol toxic effects include inhibiting SGLT-1 in the brush border membrane in the small intestine, which limits glucose absorption [48]. The decreased glucose uptake was demonstrated to be caused by lower expression of the SGLT1 [49], as well as an inhibitor of the transporter [50].

At the cellular level, deoxynivalenol has shown impairment on the translation of mRNA that may ultimately affect cell proliferation, development, and death [51–54] and reduction in pig feed intake and growth [40,55]. Also, deoxynivalenol may impair the Wnt/β-catenin pathway resulting in reduced mitosis in cells of the intestinal crypts [56]. As a result, the lower energy and nutrient intake and nutrient absorption along with impaired cell metabolism negatively impacted pig growth [57].

Besides the aforementioned toxic effects, deoxynivalenol can debilitate liver and kidney function [35,58]. Deoxynivalenol may also suppress the immune system at high doses, or stimulate the immune system at low doses [51]. In pigs chronically fed deoxynivalenol contaminated diets, it is observed increased expression of interleukin-8 and glutathione peroxidase [55], and increased serum total immunoglobulin A and specific immunoglobulin G [59]. A summary of studies showing the toxic effects of deoxynivalenol on the growth performance of pigs is shown in Table 2.

Table 2. Toxic effects of deoxynivalenol challenge, alone or in combination with other mycotoxins, on growth performance of nursery pigs.

Mycotoxin, mg/kg	n	BW, kg	Duration, d	Change, %			Reference
				ADG	ADFI	G:F	
DON, 1 (purified)	120	10 to 20	23	-0.4	-1.1	-1.9	[60]
DON, 2.3	36	7.5 to 16.5	21	-18.4	-15.9	-4.2	[57]
DON, 2.6 (purified)	120	7 to 10	14	-12.2	-11.3	0.0	[60]
DON, 3.2	60	8.2 to 20.6	34	-11.7	-5.9	-5.6	[12]
DON, 3.5 (purified)	16	8 to ?	35	-19.2	-19.8	+0.8	[61]
DON, 3.55	24	6 to 11	21	-17.8	-14.6	-3.8	[62]
DON, 4.2	126	13.4 to 22.4	21	-18.9	-12.0	-7.8	[63]
DON, 4.61	20	6.9 to 11.0	14	-41.0	-21.5	-23.7	[64]
DON, 7.38 (purified)	10	19.3 to 40.1	28	-30.2	-7.1	-24.8	[36]
AFL, 0.18; FUM, 9; DON, 1	48	6 to 29	36	-15.8	-18.5	+2.9	[65]
DON, 4.45; FB1, 0.76; ZEA, 0.44	780	22.8 to 103.8	115	-12.0	-8.7	-2.4	[40]

ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ration; BW, body weight; AFL, aflatoxins; DON, deoxynivalenol; FUM, fumonisins; ZEA, zearalenone; FB1, fumonisin B1.

1.4.3. Fumonisins

The main representant of the group composed of fumonisins is fumonisin B1. Fumonisin B1's toxic effects are due to the inhibition of ceramide synthase, resulting in impaired sphingolipid metabolism with an accumulation of sphinganine [66]. The increase in sphinganine concentration in the liver and kidney is associated with induced cell apoptosis and mitosis leading to fibrosis and nodular hyperplasia, respectively [67,68]. Therefore, the sphinganine to sphingosine ratio is frequently investigated as a biomarker for fumonisin B1 intoxication.

In pigs, fumonisin B1 intoxication is associated with lung edema. The toxicosis can be observed within one week of feeding fumonisin B1 contaminated diet where respiratory distress and cyanosis signals are observed and may evolve to death [69]. Other effects of fumonisin B1 intoxication include cellular and humoral immunosuppression [70,71], hyporexia, and decreased weight gain [72]. In the gastrointestinal tract, fumonisin B1 disrupts the intestinal barrier, by

affecting tight junction function [73]. Altogether, the impaired immune and barrier function, turn the intoxicated pig more susceptible to opportunistic pathogens [74].

1.4.4. Multiple mycotoxin toxicity

In the swine industry, multiple mycotoxins are detected, partially due to using a variety of feedstuffs in the finished feed and partially due to multiple fungi contamination. Therefore, pigs are more likely to face multiple mycotoxin toxicity in commercial farms than being challenged with a single mycotoxin. Animals, in general, are more sensitive to the toxic effect of mycotoxins when they are young and the pig is the domestic species showing the highest toxicity to multiple mycotoxins, for instance, aflatoxins, deoxynivalenol, and fumonisins [75–77]. Overall, the toxic effect is stronger when mycotoxins are co-contaminants, even if levels below the advisory guidelines.

In the review prepared by Alassane-Kpembi et al. [78], the interaction among mycotoxins is compared across *in vitro* toxicological studies. The concomitant challenge with aflatoxins (B1, B2, M1, and M2) showed a synergistic toxic effect, whereas aflatoxin B1 in combination with fumonisin B1 showed antagonistic carcinogenic effect but synergistic immunotoxic effect. For the interaction among aflatoxin B1 and trichothecenes, observed effects are either synergistic or additive. In porcine kidney cells, aflatoxin B1 and deoxynivalenol showed synergistic cytotoxic damages to incubated cells [79]. Among trichothecenes, the interactive effects seem to be variable depending on doses and proportions. In human intestinal cells, the combination of deoxynivalenol with its acetylated forms may result in synergistic or additive (3-acetyl-deoxynivalenol) to antagonistic (15-acetyl-deoxynivalenol) effect for low or high concentrations, respectively [80]. A similar study was conducted with intestinal porcine cells, where all trichothecenes mixtures showed a higher inhibitory effect than the single mycotoxins [81].

Specifically for deoxynivalenol and 3-acetyl-deoxynivalenol, low, intermediate, and high doses presented respectively antagonistic, additive, and synergic effects [81]. In intestinal porcine cells exposed to deoxynivalenol, fumonisin B1, and zearalenone, there was an additive cell toxicity, whereas deoxynivalenol and zearalenone had a synergic inhibitory effect on cell proliferation [78]. *Ex vivo* results in jejunal porcine explants show a strong (2 to 14-fold increase) synergic effect of deoxynivalenol and nivalenol regarding inflammatory cytokine expression [82].

Alike *in vitro* and *ex vivo* outcomes, mixtures of aflatoxins, deoxynivalenol, and fumonisins have also shown different effects in animals, where mostly additive and synergistic effects were observed. In *in vivo* studies, aflatoxins and deoxynivalenol together cause liver damage and impair immune function resulting in decreased growth in pigs [83,84]. Reinforcing the hypothesis of liver damage caused by mycotoxins, such mycotoxins reduced blood serum cholesterol in pigs fed mycotoxin contaminated diet [57]. Mycotoxin challenge with deoxynivalenol and aflatoxin B1 reduces apparent ileal digestibility of nutrients in feeds in newly-weaned pigs [57]. Additive or synergistic effects of deoxynivalenol and zearalenone were reported for parameters of immune function in mice and pigs [85].

In a meta-analysis prepared by Grenier and Oswald [86], publications were assessed for mycotoxin interactions. It was observed that aflatoxins and fumonisins showed mostly a synergistic effect in reducing feed intake and weight gain of pigs. For aflatoxins in combination with deoxynivalenol, it was observed a synergistic effect in reducing cholesterol and glucose and in increasing white blood cells, whereas there was an additive effect on creatine phosphokinase reduction but less than additive effect on reducing weight gain. Lastly, the interaction between fumonisins and deoxynivalenol showed a synergistic effect on decreasing weight gain and increasing hepatic enzymes, but additive for reducing feed intake.

1.4.5. Estimation of multiple mycotoxin toxicity

When using naturally contaminated feedstuffs in research trials, pigs are likely to be challenged with multiple mycotoxins although one is of main interest and/or is above the advisory guidelines. Thus, this section aimed to estimate the individual contribution of mycotoxins in a multiple mycotoxin challenge. Based on the published mycotoxin studies performed by our research group [12,35,57,65,83,84,87,88], it was possible to estimate parameters that would influence percentual changes in growth performance variables in pigs under multiple mycotoxin toxicity (Figure 1). The reason for choosing the studies performed by our research group is due to similarities in pig genetics, environment (research facilities), and feedstuffs used (sometimes the same across studies). The candidate parameters included in the selection procedure for finding the best model were: supplemental mycotoxin concentrations (mg/kg) for deoxynivalenol, aflatoxins, zearalenone, and fumonisins, as well as average initial body weight (kg) in challenged and non-challenged pigs, phase (either nursery or grower), and duration of the challenge period in days. The supplemental mycotoxin concentrations used were the differential concentration among diets of pigs challenged or not with mycotoxins within each study. The selection of parameters was performed with the GLMSELECT procedure of SAS (version 9.3, Cary, NC, USA) using the STEPWISE statement. Then, the estimates for the selected parameters were obtained with the REG procedure. The estimations generated were based on supplemental mycotoxin concentration ranging from 0 to 4.46 mg/kg for deoxynivalenol, from 0 to 0.22 mg/kg for aflatoxins, from 0 to 0.75 mg/kg for zearalenone, from 0 to 14 mg/kg for fumonisins, and from 21 to 48 days of challenge period.

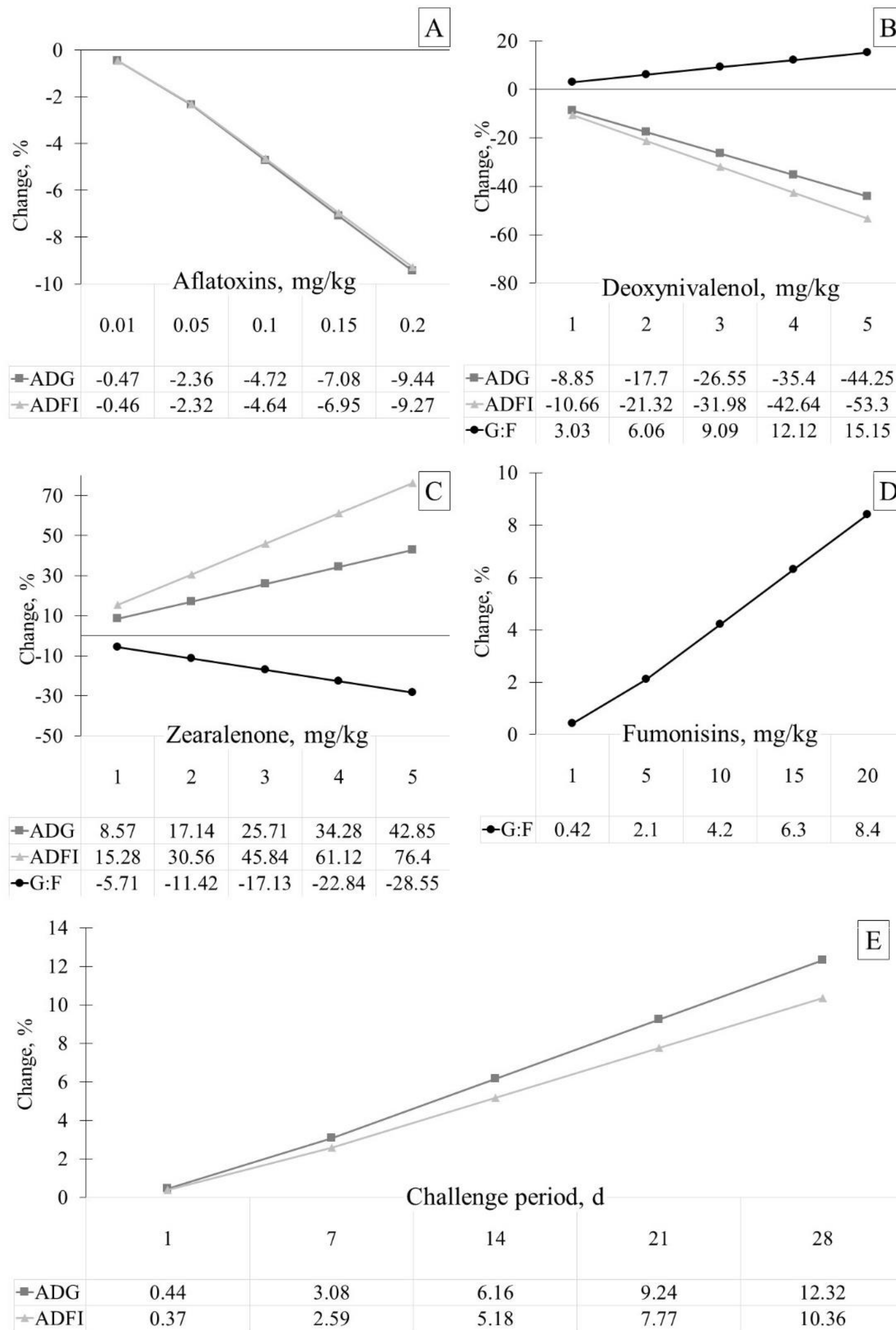

Figure 1. Parameter estimates of regression models for percentual changes in growth performance variables in pigs challenged with multiple mycotoxins. **A**, Percentual changes in average daily gain (ADG) and average daily feed intake (ADFI) caused by supplemental aflatoxins in the feed when all other variables remain constant. **B**, Percentual changes in ADG, ADFI, and gain to feed ratio (G:F) caused by supplemental deoxynivalenol in the feed when all other variables remain constant. **C**, Percentual changes in ADG, ADFI, and G:F caused by supplemental zearalenone in the feed when all other variables remain constant. **D**, Percentual changes in G:F caused by supplemental fumonisins in the feed when all other variables remain constant. **E**, Percentual changes in ADG and ADFI caused by increasing days of the challenge period when all other variables remain constant.

The results obtained showed that the increase in body weight of pigs (BW) during the study period was diminished (-8.8%) by supplemental 1 mg/kg of deoxynivalenol in feed (DON) and supplemental 0.01 mg/kg of aflatoxins in the feed (AF; -0.4%), but it was increased by supplemental 1 mg/kg of zearalenone in the feed (ZEA; +8.5%) and duration in days of the challenge (day; +0.4%), whereas there was no influence of supplemental fumonisins in feed (FUM), initial body weight in non-challenged pigs, initial body weight in challenged pigs, phase or. The adjusted R-square for the equation (BW = -15.0+ DON*-8.8 + AF*-0.4 + ZEA*8.5 + day*0.4) generated was 0.83 ($p < 0.001$).

The average daily gain (ADG) of pigs was diminished (-8.9%) by supplemental 1 mg/kg of deoxynivalenol and supplemental 0.01 mg/kg of aflatoxins (-0.5%), but it was increased by supplemental 1 mg/kg of zearalenone (+8.6%) in feed, and each day of challenge duration (+0.4%), whereas there was no influence of supplemental fumonisins in feed, initial body weight in non-challenged pigs, initial body weight in challenged pigs, or phase. The adjusted R-square for the equation (ADG = -15.6+ DON*-8.9 + AF*-0.5 + ZEA*8.6 + day*0.4) generated was 0.83 ($p < 0.001$).

The average daily feed intake (ADFI) of pigs was diminished by supplemental 1 mg/kg of deoxynivalenol (-10.7%) and supplemental 0.01 mg/kg of aflatoxins (-0.5%) in feed, but it was increased by supplemental 1 mg/kg of zearalenone (+15.3%) in feed and each day of challenge (+0.4%), whereas there was no influence of supplemental fumonisins, initial body weight in non-challenged pigs, initial body weight in challenged pigs, or phase. The adjusted R-square for the equation (ADFI = -9.1+ DON*-10.7 + AF*-0.5 + ZEA*15.3 + day*0.4) generated was 0.86 ($p < 0.001$).

The gain to feed ratio (GF) of pigs was diminished by supplemental 1 mg/kg of zearalenone (-5.7%) in the feed but it was increased by supplemental 1 mg/kg of deoxynivalenol (+3.0%) and supplemental 1 mg/kg of fumonisins (+0.4%) in feed, whereas there was no influence of supplemental aflatoxins, initial body weight in non-challenged pigs, initial body weight in challenged pigs, phase, or days of the challenge. The adjusted R-square for the equation (GF = -6.7 + DON*3 + FUM*0.4 + ZEA*-5.7) generated was 0.48 ($p = 0.013$).

The observed decreases in body weight gain, average daily gain, and average feed intake caused by deoxynivalenol and aflatoxins were expected because of the toxic effects of these mycotoxins in pigs, as mentioned before, impairing cell metabolism, nutrient utilization, and pig growth. Deoxynivalenol and fumonisins increased the gain to feed ratio, increasing the efficiency of the conversion of nutrients into body tissues. The improved efficiency may happen as a result of reduced body weight and feed intake in challenged pigs, which may become more efficient in using nutrients from feed [89]. Even though the increase in body weight gain, average daily gain, and average daily feed intake caused by zearalenone is controversial, similar outcomes were previously reported in studies with the purified toxin along with a decrease in gain to feed ratio [90,91]. The lack of significant effect of fumonisins on body weight gain, average daily gain, and average daily feed intake could be due to the average concentration of fumonisins across studies, which was 2 mg/kg. Indeed, such a concentration is below the advisory guidelines published in the United States [27] and Europe [7] and, thus, may explain the absence of detrimental effects in pigs in the model. Unexpectedly, it was observed that an increase in the number of days of mycotoxin challenge actually increased body weight gain, average daily gain, and average daily feed intake. This result could be because of the challenge period included in the model (from 21

to 48 days), when pigs are facing the chronic effects of mycotoxins. Therefore, an increase in days could reduce the toxic effects of mycotoxins as pigs may get acclimated to mycotoxins.

Altogether, the elevated prevalence of mycotoxins (aflatoxins, deoxynivalenol, and fumonisins), their toxic effects, and the existing advisory levels stipulated by governmental institutions for pigs turn valuable the researches assessing the efficiency of feed additives that can be used as mycotoxin mitigators.

1.5. Dietary mitigation additives for mycotoxin toxicity

Aiming to enable the use of contaminated feedstuffs and feeds for animal consumption by diminishing or avoiding the toxic effects of mycotoxins, dietary mitigation additives can be employed. There are many dietary mitigators of mycotoxin toxic effects with different mechanisms of action. For example, there are several sources of additives attempting to mitigate the toxic effects of mycotoxins in pigs by adsorption, enhancing immune function, and as detoxifiers (such as microorganisms) [92,93]. Particularly for deoxynivalenol, it is still a challenge to find an efficient feed additive able to counteract its toxic effects. In Table 3, the effects of dietary additives on growth performance of pigs challenged with deoxynivalenol, alone or in combination with other mycotoxins, are summarized.

Table 3. Effects of dietary additives on growth performance of pigs challenged with deoxynivalenol, alone or in combination with other mycotoxins.

Mycotoxin, mg/kg	n	BW, kg	Duration, d	Additive			Change, %			Reference
				Inorganic	Yeast	Other	ADG	ADFI	G:F	
DON, 1	120	10 to 20	23	Acid-activated bentonite, clinoptilolite	Yeast cell wall	Organic acids	+5.0	+4.7	0.0	[60]
DON, 2.3	36	7.5 to 16.5	21	-	Hydrolyzed yeast cell wall	Organic acids, vitamins, and essential oils	+5.4	+5.6	+9.0	[57]
DON, 2.6	120	7 to 10	14	Acid-activated bentonite, clinoptilolite	Yeast cell wall	Organic acids	+28.9	+19.8	+7.5	[60]
DON, 3.2	36	8.19 to 20.73	34	Bentonite	Yeast culture	Diatomaceous earth, kelp	+11.8	+4.7	+6.0	[12]
DON, 3.2	36	8.19 to 20.55	34	Organo-aluminosilicate clays	yeast cell walls	Plant extracts, triglycerides, calcium propionate, and antioxidants	+7.4	+3.1	+4.5	[12]
DON, 3.2	36	8.21 to 20.44	34	Sepiolite and bentonite	Inactivated yeast and fermentation extracts	Propyl gallate, calcium propionate, milk thistle seed, rosemary, licorice root, and boldo	+4.1	-0.8	+3.0	[12]
DON, 3.82	30	6.9 to 11.2	14	-	-	Sodium metabisulfite, organic acids, vitamins, and amino acids	+60.0	+13.8	+36.2	[64]

Table 3. (Continued).

Mycotoxin, mg/kg	n	BW, kg	Duration, d	Additive			Change, %			Reference
				Inorganic	Yeast	Other	ADG	ADFI	G:F	
DON, 4.2	126	13.4 to 22.4	21	Adsorbent clays	-	Preservatives	-10.1	-9.0	-1.6	[63]
DON, 4.41	30	7.0 to 10.9	14	-	Yeast extract	Live bacteria, enzymes, plant extracts	+19.5	+12.8	+5.2	[64]
DON, 4.66	30	6.9 to 10.6	14	-	Yeast glucomannan	-	+1.8	+6.9	-8.6	[64]
DON, 4.65	30	6.9 to 10.7	14	Aluminosilicate	-	-	+7.3	0.0	+6.9	[64]
DON, 4.45; FB1, 0.76; ZEA, 0.44	780	22.9 to 104.6	115	-	-	Sodium metabisulfite, organic acids, vitamins, and amino acids	+9.1	+5.6	+3.0	[40]
DON, 4.45; FB1, 0.76; ZEA, 0.44	780	22.8 to 103.3	115	Hydrated sodium calcium aluminosilicate, silicon dioxide	Hydrolyzed yeast	-	+4.5	+3.4	+0.8	[40]
AFL, 0.18; FUM, 9; DON, 1	48	6 to 29	36	Hydrated sodium-calcium aluminosilicate	Yeast cell wall	Algae	0.0	-3.9	+4.3	[65]

ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ration; AFL, aflatoxins; DON, deoxynivalenol; FUM, fumonisins; ZEA, zearalenone; FB1, fumonisin B1.

1.5.1. Inorganic compounds

The activated charcoal is known for a long time as a potent adsorbent for multiple mycotoxins [94,95]. However, because of its nonspecific binding (inclusive of nutrients in feed), the activated charcoal should have its use restrained to cases of acute intoxication with high concentrations of mycotoxins when there is an imminent risk of severe toxicosis or death [96].

Aluminosilicates have a comparatively lower adsorbability to mycotoxins, but it is considerably enhanced for the hydrated sodium-calcium form, particularly to aflatoxins [97], or in case aluminosilicates are in association with organic compounds [98]. The adsorbability of several inorganic adsorbents to aflatoxin B1 was tested in an *in vitro* model, where a carbon and aluminosilicate-based product, phyllosilicates (Attapulgite, Greek bentonite, sodium bentonite, activated bentonite, Indian bentonite, Myco AD A-Z), and tectosilicates (Clinoptilolite, CAB 70) were tested [98]. At 1 and 10 µg/mL of aflatoxin B1, the carbon and aluminosilicate-based product and phyllosilicates showed binding efficiency of approximately 80% or more to aflatoxin B1, whereas a lower efficiency (61 to 8%) was observed for tectosilicates. *In vivo*, bentonites and hydrated sodium-calcium aluminum silicates could effectively overcome the toxic effects of aflatoxins in pigs [99], even though they may also interact with minerals in feed [33].

Bentonites show low adsorbability for deoxynivalenol (3.2%) *in vitro* in comparison to other mycotoxins as aflatoxins (92.5%) [100]. The higher polarity of aflatoxins when compared to deoxynivalenol is likely the cause for the reduced binding ability of bentonites to deoxynivalenol [97,101]. Diatomaceous earth has an intermediate adsorbability to mycotoxins, still, it is amid the inorganic materials with the highest adsorbability considering *Fusarium* mycotoxins [97].

A carbon and aluminosilicate-based product, the same as aforementioned, was tested in a different study for fumonisin B1 binding, showing higher adsorbability among all mycotoxins tested under different pHs (100%) [102]. Fumonisin B1 adsorption by other aluminosilicates (bentonite and zeolite) and diatomaceous earth (celite) showed higher efficiencies in an acidic environment up to 100% (bentonite) but was greatly decreased in a neutral environment to 26% (bentonite) [102].

1.5.2. Yeast

The use of yeast in the livestock feed industry emerged from the abundance of by-products from the food industry. One of such uses of yeast as a feed additive is as a mycotoxin adsorbent. Cellular components from yeasts, the cell wall and intracellular content, may be used as feed additives. The cell wall is a complex structure of carbohydrates composed of glucans, mannans, and chitin [103,104]. Out of the carbohydrates composing the yeast cell wall, glucans were identified as a fundamental element in the interaction and inactivation of mycotoxins [65,105,106] and prebiotic properties [107,108]. The α- and β-D-glucans are elements of the yeast cell wall which may selectively interact with enterocytes and microbes modulating immune function and microbiome, respectively [109]. Yeast cell wall interaction with enterocytes and microbes has further effects, resulting in diminished oxidative stress [110,111]. Therefore, dietary yeast cell wall can have advantageous prebiotic properties by facilitating the metabolism and growth of beneficial microorganisms, resulting in improved intestinal barrier, health, and immunity [112,113]. Also, the inclusion of yeast culture, as probiotic, may increase the carbohydrate fermentation in the intestinal lumen providing beneficial metabolic products, as peptides and organic acids, and improving the nutrition and health of pigs [108]. The feed additives containing fermentation extracts derived from yeast metabolism may have similar

beneficial effects as yeast metabolic products [12]. Nevertheless, the fermentation extracts will be present in a limited amount in the additive instead of being produced in the intestinal lumen. The use of inactivated yeast may enable yeast cell wall interaction with enterocytes and with mycotoxins, similarly to using yeast culture or yeast cell wall extract, improving intestinal health and reducing mycotoxin toxicity. Enhanced intestinal health and immune response are seen after the inclusion of a feed additive with *Saccharomyces cerevisiae* as either yeast culture or inactivated yeast [12,57], through a reduction in CD4+ activation and eventual IFN-γ production [108,114], thus, reducing inflammation and enhancing enterocyte integrity [115]. Feed additives with yeast culture have shown enhanced animal health, gut integrity, and digestibility of nutrients in feed, along with decreased *Escherichia coli* shedding in feces, improving the performance of non-challenged pigs [108,114].

The yeast cell wall, and more particularly β-D-glucans, have robust adsorbability to aflatoxin B1 and zearalenone but with restricted efficiency to deoxynivalenol [100,106]. The detoxification of aflatoxin B1 by *Saccharomyces cerevisiae* strains was, on average, 65% after 24 hours of incubation [116].

Yeast- and algae-derived β-glucans can show higher adsorbing abilities than mineral adsorbents, especially under alkaline pH for deoxynivalenol and zearalenone [101,106,117]. However, this limited (but existing) adsorbability of yeast cell wall components as β-D-glucans and glucomannans to *Fusarium* toxins [118,119] can be an advantage in comparison to inorganic binders. Indeed, in an *in vitro* study simulating the gastrointestinal tract of pigs, yeast cells could adsorb 23% of deoxynivalenol whereas bentonite, cellulose, and activated charcoal could adsorb 3, 12, and 14% of deoxynivalenol, respectively [100]. In another study, the detoxification of deoxynivalenol by *Saccharomyces cerevisiae* strains was, on average, 33% after 24 hours of

incubation [116]. Yet low, the binding ability of the yeast cell wall seems to be the highest towards deoxynivalenol. In addition, processing yeast into yeast cell walls may result in improved adsorbability to mycotoxins [97]. There are few investigations of the yeast cell wall as a sole dietary additive to mitigate the toxic effects of deoxynivalenol in pigs. Yeast cell wall's minor effects in ameliorating health and growth in deoxynivalenol challenged pigs is likely the reason for the small number of studies [40,120], as the yeast cell wall plays an accessory effect as a deoxynivalenol mitigator. Specifically related to the deoxynivalenol challenge, the yeast cell wall seems to have lower immune-modulatory effects than the whole cell in newly-weaned pigs, indicating that yeast fermentation products may have a major function in mitigating the toxic effects of deoxynivalenol in the gut in comparison to the yeast cell wall [12]. Such an outcome could be due to reduced immune response and improved maintenance of gut integrity, both with a major role in pigs' susceptibility to deoxynivalenol instead of the adsorbability of yeast fermentation products [12].

In contrast to deoxynivalenol, the detoxification of fumonisins (another *Fusarium* toxin) by *Saccharomyces cerevisiae* strains was, on average, 72% after 24 hours of incubation [116]. However, the detoxifying capacity of yeast is not high for all species and strains. Naturally occurring bacteria and yeast in silage were tested for fumonisin detoxifying capacity, where bacteria showed up to 5-fold higher detoxification in comparison to yeast [121]. In most studies including fumonisins and yeast-based products, other mycotoxins are co-contaminants [57,65,87]. Probably, the lack of studies is due to the high efficiency of additives with other components, as discussed above in the case of inorganic adsorbents. Only one *in vivo* study assessing yeast mitigation effects in fumonisin challenged pigs as a single toxin was found in our survey of scientific publications. A recent study was found where pigs were challenged with

fumonisins and 3 different products, one of them was a yeast-based product and showed a recovery in growth performance and sphinganine to sphingosine ratio [122]. Nevertheless, the other 2 products tested were not specific for fumonisins.

1.5.3. Bacteria

Similarly, the investigation of alternative uses of by-products rich in bacteria emerges from the dairy and baking industries. The binding ability of *Lactobacillus casei* to aflatoxin B1 was shown to depend on the cell wall structure, where the live cell or cell wall fractions had similar adsorbability but heat treatment decreased its adsorbability [123]. In this case, the damage to the protein structure, by which aflatoxin B1 has a high affinity, by heat treatment was claimed as the reason for *L. casei* losing its adsorbability. The adsorption of aflatoxin B1 by live cells of *L. casei* (10^9 CFU/day) caused conformational changes in the bacterial cell wall, reduced the intestinal absorption of aflatoxin B1, and overcame the detrimental effects observed in mice [123]. The detoxification of aflatoxin B1 by *Lactobacillus* species was, on average, 60% after 24 hours of incubation [116].

Feeding deoxynivalenol to weanling pigs may modulate the gastrointestinal microbiome [124], indicating that the gastrointestinal microbiome can undergo changes to avoid deoxynivalenol toxicity. Among microbial genera with deoxynivalenol detoxifying capacity, are Eubacteria, *Anaerofilum*, *Collinsella*, *Bacillus*, and Clostridiales [125]. Least commonly but also of interest, aerobic gram-positive bacteria may catalyze the de-epoxidation reaction as described for *Nocardioides* and even aerobic gram-negative as *Devosia*, being generally characterized as casual degraders [126]. The detoxification of deoxynivalenol by *Lactobacillus* species was, on average, 30% after 24 hours of incubation [116]. However, it was observed that additives with gram-positive bacteria can adsorb deoxynivalenol rather than converting it to its less toxic

compounds [127]. Gram-positive bacteria as *Streptococcus* and *Enterococcus* have shown adsorbability achieving up to 33% towards deoxynivalenol in corn silage [127], whereas *Lactobacillus helveticus* could adsorb 55%, and heat-inactivated *Lactobacillus plantarum* could adsorb up to 71% of deoxynivalenol in liquid media [128]. Following up on this study, the adsorbability of deoxynivalenol by several gram-positive bacteria was tested and an overall increase in the adsorbability was observed after heat treatment [128]. Such lack of species-specificity adsorption across gram-positive bacteria strains along with the increased adsorption after heat-inactivation of bacteria suggest that the bacterial cell wall may be involved in the mycotoxin-bacteria interaction. The role of the cell wall from gram-positive bacteria in the adsorption of deoxynivalenol was later proved by Zou et al. [129], where similar adsorption by *L. plantarum* was demonstrated by either the cell pellet or cell wall, but no adsorption was observed by the cell extract or its fermentation product. Of interest, the deoxynivalenol-*L. plantarum* interaction could be preserved when incubated in simulated gastric and intestinal fluids for 30 minutes to 4 hours [129]. The detailed mechanism by which deoxynivalenol adsorption occurs by the cell wall of gram-positive bacteria is not fully elucidated. But it can be inferred that the increase in temperature causes protein denaturation leading to pore formation, which could enhance the surface area with the binding ability [130]. Moreover, the hydrophobicity of the cell wall from *Lactobacillus* [130] is enhanced by heat treatment [131], which may facilitate the deoxynivalenol adsorption [129]. *Ex vivo*, the culture supernatant from *L. plantarum* after heat treatment could improve the architectural damages to intestinal villi caused by deoxynivalenol in jejunal explants of pigs [132]. Similarly, jejunal explants of pigs treated with *Lactobacillus rhamnosus* (10^9 CFU/mL) before deoxynivalenol exposure showed a reduction in paracellular permeability, production of pro-inflammatory cytokines (tumor necrosis

factor-α and interleukin-8), and activation of mitogen-activated protein kinases [133]. Although, due to deoxynivalenol's small chemical structure and low polarity, finding compounds with strong adsorbability to deoxynivalenol and with the potential to mitigate its toxic effects is a current challenge [63,64,101].

Following the same line, the mechanism of interaction between fumonisins and the cell wall from gram-positive bacteria was investigated. The adsorbability of fumonisins by the cell wall from gram-positive bacteria increases with a further breakdown of the cell wall structure as long as the peptidoglycan remains intact [134]. In a later study, the peptidoglycan was recognized as the component from the bacterial cell wall which adsorbs the tricarballylic acid chain from fumonisins [134]. The detoxification of fumonisins by *Lactobacillus* species was about 70% after 24 hours of incubation [116]. However, no *in vivo* studies were found testing bacterial mitigating properties in pigs challenged with fumonisins as a single mycotoxin. It is likely that the absence of studies with bacterium-based products in pigs challenged with fumonisins, as seen for yeast-based products, is because of the high efficiency of inorganic adsorbents.

1.5.4. Others

Plant extracts and antioxidants are often included in mycotoxin toxicity mitigators formulas to diminish the oxidative stress caused by mycotoxins and enhance intestinal health. Algae derivatives may present antioxidant properties under mycotoxin challenge, resulting in an enhanced gain in poultry [135]. In pigs, algae extract improved nutrient and energy digestibility, decreased *E. coli* counting in feces, and improved growth performance [136]. Feed additives containing calcium propionate may reduce intestinal pH, and increase the digestibility of nutrients in feed and improve intestinal health [137]. Also, calcium propionate is an organic acid

with the ability to impair fungal colonization or growth in feeds [75]. Considering animals under mycotoxin challenge, calcium propionate improved liver health, reduced mycotoxin concentration in organs, and recovered the growth performance in broilers [138]. In pigs, a mycotoxin mitigator combining hydrated-sodium calcium aluminum silicate, calcium propionate, and calcium formate was tested to mitigate the toxic effects of multiple mycotoxins (zearalenone, aflatoxin, and ochratoxin) promoted intestinal health, nutrient digestibility and absorption, and gain [139]. Plant derivatives, like milk thistle, can reduce inflammatory signaling *in vitro* by reducing tumor necrosis factor-alpha and, resulting in reduced cell death [140]. Another example is rosemary which may neutralize and destroy *Fusarium* fungi [141].

Overall, the use of feed additives to mitigate the toxic effects of mycotoxins containing multiple components has shown more benefits in comparison to those with single components, particularly in the case of deoxynivalenol challenge [115].

1.6. Conclusion

In conclusion, mycotoxins have a high prevalence in feedstuffs and swine feeds globally which may impair pig health and growth. However, it is also important to consider the co-occurrence of mycotoxins and the occurrence of emerging mycotoxins as well as in their masked form. It is advisable for future studies to have a broad mycotoxin screening enabling correct interpretation of data and projection of trends. Dietary mitigation additives include adsorbents, health stimulants, and detoxifiers. The frequent co-contamination with mycotoxins of feedstuffs and feeds intended for pig consumption turns mitigation additives targeting multiple mycotoxins preferable.

1.7. Scope of current research

Mycotoxins are a current concern globally. The contamination of feed ingredients with mycotoxins is a fact that may get worse shortly with increasing frequency or contamination levels of mycotoxins due to global warming. After their ingestion, mycotoxins may lead to detrimental effects in livestock, reducing feed intake, modulating immune function, causing oxidative stress, impairing cell metabolism, and may culminate in cell death. As a result, mycotoxins impair animal health and growth.

Weaning stress is caused by changes in several factors including physical and social environments of the suckling pig. Another change that directly influences gut health and functions is the sudden transition from the liquid and milk-based diet to a solid and plant-based diet. Such dietary challenge can be potentiated in case mycotoxins are present in the feed. Additionally, the pig is the most sensitive domestic species with increased susceptibility to mycotoxin detrimental effects at early ages.

Pig feed is commonly found to be contaminated with at least one mycotoxin and the most frequently detected are deoxynivalenol, fumonisins, and aflatoxin. Feed additives aiming to adsorb to mycotoxins, enhance immunity, improve gut health, or convert mycotoxins to a less toxic form can be used to diminish mycotoxin detrimental effects in pigs. Out of the three most frequent mycotoxins, aflatoxins and fumonisins already have efficient feed additives that can be used to counteract mycotoxin detrimental effects. However, deoxynivalenol still lacks an efficient feed additive to overcome its detrimental effects in pigs. The yeast can be an alternative feed additive as its cell wall show higher adsorbability to deoxynivalenol than inorganic sources. At the same time, feed supplementation with yeast culture and yeast metabolites has shown to

improve gut immunity, health, and functionality, resulting in reduced stress effects and enhanced pig growth.

Thus, this research focused on investigating the use of yeast-based products as a feed additive to mitigate deoxynivalenol detrimental effects on growth and health of newly-weaned pigs.

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CHAPTER 2. INVESTIGATION OF THE EFFICACY OF A POSTBIOTIC YEAST CELL WALL-BASED BLEND ON NEWLY-WEANED PIGS UNDER A DIETARY CHALLENGE OF MULTIPLE MYCOTOXINS WITH EMPHASIS ON DEOXYNIVALENOL

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2.1. Abstract

Pigs are highly susceptible to mycotoxins. This study investigated the effects of a postbiotic yeast cell wall-based blend (PYCW; Nicholasville, KY, USA) on growth and health of newly-weaned pigs under dietary challenge of multiple mycotoxins. Forty-eight newly-weaned pigs (21 d old) were individually allotted to four dietary treatments, based on a three phase-feeding, in a randomized complete block design (sex; initial BW) with two factors for 36 d. Two factors were dietary mycotoxins (deoxynivalenol: 2,000 µg/kg supplemented in 3 phases; and aflatoxin: 200 µg/kg supplemented only in phase 3) and PYCW (0.2%). Growth performance (weekly), blood serum (d 34), and jejunal mucosa immune and oxidative stress markers (d 36) data were analyzed using MIXED procedure of SAS. Mycotoxins reduced ($p < 0.05$) average daily feed intake (ADFI) and average daily gain (ADG) during the entire period whereas PYCW did not affect growth performance. Mycotoxins reduced ($p < 0.05$) serum protein, albumin, creatinine, and alanine aminotransferase whereas PYCW decreased ($p < 0.05$) serum creatine phosphokinase. Neither mycotoxins nor PYCW affected pro-inflammatory cytokines and oxidative damage markers in the jejunal mucosa. No interaction was observed indicating that PYCW improved hepatic enzymes regardless of mycotoxin challenge. In conclusion, deoxynivalenol (2000 µg/kg, for 7 to 25 kg body weight) and aflatoxin B1 (200 µg/kg, for 16 to 25 kg body weight) impaired growth performance and nutrient digestibility of newly-weaned pigs, whereas PYCW could partially improve health of pigs regardless of mycotoxin challenge.

Keywords: aflatoxin; deoxynivalenol; pig; yeast

2.2. Introduction

Mycotoxins are fungal secondary metabolites, which may have deleterious effects when consumed. Mycotoxins are naturally present in several plants and products that are used as feedstuffs worldwide, where aflatoxin B1 (AFB1) and deoxynivalenol (DON) are considered major contaminants [1,2]. In the past decade, *Fusarium* toxins were the most prevalent mycotoxins worldwide, where DON ranked first position with 64% of feedstuff samples testing positive, whereas AFB1 was the most frequent among non-*Fusarium* toxins with 23% of samples testing positive [3].

As extensively reviewed [4–6], the pig is the most sensitive species to multiple mycotoxins, more specifically, aflatoxins, deoxynivalenol, zearalenone, and fumonisins especially in the early stages of production. Among these, aflatoxins and DON combined decrease pig growth, lead to liver damage, reduce enterocyte viability, and compromise immune function [7–9]. Aflatoxin B1 and DON present greater cytotoxicity and have caused synergistic damaging effects in porcine kidney cells [10]. Due to the high prevalence and detrimental effects of AFB1 and DON, regulatory limit levels have been established worldwide. For instance, aflatoxin is limited at 20 [11] or 10 μg/kg of feed [12] for growing pigs, whereas DON has advisory levels of 1000 [13] or 900 μg/kg of feed [14] established by the Food and Drug Administration or European Commission, respectively. Thus, there are several strategies attempting to mitigate negative effects of mycotoxins in animals, such as the implementation of prebiotics, probiotics, and adsorbents [15,16].

Components from yeast organisms, subdivided into parietal (cell wall) and intracellular content, can be used as feed additives. The cell wall consists of a carbohydrate network made of glucans, mannans and chitin [17,18], the former having been identified as key components in

mycotoxin deactivation [19–21] and prebiotic properties [22,23]. The postbiotic yeast cell wall, and more specifically β-d-glucans composing the inner layer portion of this network, have strong adsorbing affinity against AFB1 or zearalenone, but have limited effectiveness toward deoxynivalenol [21,24]. In addition, certain yeast cell wall components, when specifically isolated and purified, can be advantageous as a prebiotic by favoring proliferation of beneficial microorganisms, enhancing gut barrier function, and supporting intestinal health and immune function [25,26].

Therefore, it was hypothesized that use of a postbiotic yeast cell wall-based blend in diets with AFB1 and DON would enhance growth, reduce liver damage, and improve immune function of newly-weaned pigs.

This study aimed at investigating the effects of a postbiotic yeast cell wall-based blend (PYCW; Alltech Inc., Nicholasville, KY, USA) on growth and health of newly-weaned pigs under chronic dietary challenges of AFB1 (43.6 μg/kg) and DON (2163 μg/kg).

2.3. Results

In the results, diets low in mycotoxins (LM) represent those formulated only with conventional feedstuffs whereas diets high in mycotoxins (HM) represent those formulated with feedstuffs including corn dried distillers grains with solubles (DDGS) high in DON and corn high in AFB1. Experimental diets were fed to newly-weaned pigs moved to the research farm in order to start the current study right after weaning.

The mycotoxin analysis performed comparing LM vs. HM diets fed to pigs show that for phases 1 and 2 the average level of AFB1 was 1.0 μg/kg and DON (including acetylated and glycosylated forms) was 676 vs. 2258 μg/kg, respectively. Mycotoxin analyses performed

comparing LM vs. HM diets fed to pigs for phase 3 found the level of AFB1 was 1.0 vs. 44.6 µg/kg and DON (including acetylated and glycosylated forms) was 1311 vs. 4635 µg/kg, respectively. The average mycotoxin concentration in the whole experimental period for LM vs. HM was 887 vs. 3,050 µg/kg of DON.

There were no differences in initial body weight of pigs among experimental groups. Feeding HM reduced ($p < 0.05$) average daily feed intake (ADFI) for every week and for the overall period, which resulted in reduced ($p < 0.05$) average daily gain (ADG) and body weight for all periods (Table 1). The HM reduced ($p < 0.05$) ADG of pigs during the first week. There was an interaction ($p < 0.05$) for gain to feed (G/F) ratio during the first week of the study, indicating that when PYCW is added to diets, feeding HM increased ($p < 0.05$) G/F. The interaction also indicates that in pigs fed MT, the addition of PYCW tended to increase ($p = 0.062$) G/F during the first week. Feeding HM also tended to increase ($p = 0.057$) G/F during the second week. Feeding diets with PYCW tended to reduce ($p = 0.056$) G/F during the overall experimental period.

Table 1. Growth performance of newly-weaned pigs fed diets high (HM) or low (LM) in mycotoxins [1] or postbiotic yeast cell wall-based blend (PYCW) for 36 days.

Diet	LM		HM		SEM	*p* Value		
PYCW	**0%**	**0.2%**	**0%**	**0.2%**		**Diet**	**PYCW**	**Diet vs. PYCW**
Body weight, kg								
d 0	7.45	7.49	7.51	7.53	0.48	0.659	0.784	0.939
d 7	10.11	9.82	9.33	9.60	0.69	0.016	0.970	0.168
d 14	13.73	13.65	12.47	12.94	0.85	0.005	0.567	0.418
d 21	17.57	17.71	15.76	16.24	0.93	<0.001	0.475	0.692
d 28	22.39	22.31	19.79	20.32	1.29	<0.001	0.697	0.598
d 36	29.49	29.43	25.74	26.47	1.47	<0.001	0.632	0.574
ADG, kg								
Phase 1 (d 0 to 7)	0.379[a]	0.333	0.261[b]	0.296	0.039	0.001	0.815	0.081
Phase 2 (d 7 to 21)	0.533	0.563	0.459	0.474	0.024	0.001	0.346	0.750
d 7 to 14	0.518	0.547	0.449	0.476	0.031	0.017	0.320	0.980
d 14 to 21	0.549	0.580	0.470	0.472	0.038	0.018	0.667	0.708
Phase 3 (d 21 to 36)	0.794	0.781	0.665	0.682	0.039	<0.001	0.938	0.553
d 21 to 28	0.688	0.657	0.576	0.584	0.055	0.005	0.714	0.545
d 28 to 36	0.887	0.890	0.743	0.768	0.031	<0.001	0.624	0.693
Overall (d 0 to 36)	0.612	0.609	0.506	0.526	0.029	<0.001	0.647	0.547
ADFI, kg								
Phase 1 (d 0 to 7)	0.310	0.315	0.239	0.246	0.040	0.002	0.765	0.972
Phase 2 (d 7 to 21)	0.651	0.700	0.559	0.594	0.039	0.002	0.170	0.817
d 7 to 14	0.622	0.679	0.519	0.551	0.047	<0.001	0.156	0.689
d 14 to 21	0.681	0.721	0.599	0.636	0.040	0.040	0.334	0.972
Phase 3 (d 21 to 36)	1.112	1.129	0.947	1.014	0.067	<0.001	0.253	0.491
d 21 to 28	0.922	0.930	0.790	0.805	0.071	0.003	0.768	0.941
d 28 to 36	1.279	1.302	1.084	1.196	0.069	0.002	0.130	0.319
Overall (d 0 to 36)	0.777	0.804	0.658	0.701	0.048	<0.001	0.191	0.764
G/F								
Phase 1 (d 0 to 7)	1.25	1.06[b]	1.11[Y]	1.40[aX]	0.13	0.381	0.627	0.031
Phase 2 (d 7 to 21)	0.82	0.81	0.83	0.81	0.03	0.963	0.593	0.825
d 7 to 14	0.83	0.82	0.87	0.87	0.04	0.057	0.882	0.726
d 14 to 21	0.81	0.80	0.79	0.75	0.05	0.425	0.557	0.776
Phase 3 (d 21 to 36)	0.72	0.70	0.70	0.68	0.02	0.283	0.164	0.996
d 21 to 28	0.75	0.71	0.73	0.72	0.02	0.842	0.284	0.368
d 28 to 36	0.70	0.69	0.69	0.65	0.03	0.253	0.286	0.616
Overall (d 0 to 36)	0.79	0.76	0.77	0.76	0.02	0.296	0.056	0.507

There was no animal mortality during the experimental period. [1] HM has 1.0 μg/kg of aflatoxin B1 (AFB1) and 2258 μg/kg of deoxynivalenol (DON) on average for phases 1 and 2, and 44.6 μg/kg of AFB1 and 4635 μg/kg of DON for phase 3. [a,b] Means with different superscripts differ ($p < 0.05$). [X,Y] Means with different superscripts tend to differ ($0.05 \leq p < 0.10$).

Feeding HM reduced ($p < 0.05$) blood serum protein, albumin, and creatinine concentrations on day 35 (Table 2). Feeding diets with PYCW tended to decrease aspartate aminotransferase (AST; $p = 0.051$) and creatine phosphokinase (CPK; $p = 0.052$) concentrations, and blood urea nitrogen (BUN)/creatinine proportion ($p = 0.088$) in blood serum. Feeding diets with PYCW reduced ($p < 0.05$) the proportion of AST/ALT (alanine aminotransferase) and increased ($p < 0.05$) glucose concentration in blood serum of pigs. There was an interaction ($p < 0.05$) for phosphorus concentration in blood serum of pigs, indicating that when there is no PYCW, HM reduced ($p < 0.05$) phosphorus concentration. The interaction also indicates that in MT, the addition of PYCW tended to increase ($p = 0.063$) phosphorus in serum.

Table 2. Serum variables observed for serum biochemistry, and electrolytes in newly-weaned pigs fed diets high (HM) or low (LM) in mycotoxins [1] or postbiotic yeast cell wall-based blend (PYCW) for 35 days.

Diet	LM		HM		SEM	*p* Value		
PYCW	0%	0.2%	0%	0.2%		Diet	PYCW	Diet vs. PYCW
Total protein, g/dL	5.24	5.26	4.91	4.98	0.15	0.001	0.602	0.742
Albumin, g/dL	3.54	3.51	3.23	3.33	0.13	0.009	0.745	0.489
Globulin, g/dL	1.70	1.75	1.68	1.66	0.14	0.367	0.796	0.605
Albumin/Globulin	2.14	2.05	2.00	2.06	0.22	0.561	0.884	0.513
AST, IU/L	37.3	32.6	43.1	29.3	5.7	0.787	0.051	0.334
ALT, IU/L	28.1	29.3	25.8	25.3	1.7	0.050	0.833	0.562
AST/ALT	1.33	1.14	1.68	1.19	0.22	0.225	0.046	0.370
ALP, IU/L	262	246	249	229	22	0.430	0.346	0.940
CPK, IU/L	5107	2156	4330	2203	1315	0.775	0.052	0.747
Cholesterol, mg/dL	94.3	88.5	84.4	85.4	3.6	0.077	0.508	0.349
BUN, mg/dL	14.3	13.3	14.1	13.2	0.7	0.796	0.126	0.897
Creatinine, mg/dL	1.02	0.99	0.90	0.96	0.04	0.035	0.630	0.232
BUN/Creatinine	14.4	13.6	16.2	13.8	1.1	0.276	0.088	0.413
Glucose, mg/dL	106	111	103	109	2	0.193	0.015	0.829
Phosphorus, mg/dL	11.4[a]	11.0	10.3[bX]	11.0[Y]	0.4	0.019	0.612	0.034
Calcium, mg/dL	11.1	11.0	10.8	11.0	0.2	0.182	0.787	0.334
Sodium, mEq/L	148.7	146.9	149.8	146.0	1.9	0.964	0.145	0.592
Potassium, mEq/L	5.83	5.64	5.81	6.05	0.22	0.370	0.893	0.332
Na/K	26.0	26.4	26.1	24.6	0.9	0.308	0.526	0.264
Chloride, mEq/L	104.8	104.3	106.8	105.0	1.2	0.192	0.252	0.565

[1] HM has 1.0 μg/kg of AFB1 and 2258 μg/kg of DON on average for phases 1 and 2, and 44.6 μg/kg of AFB1 and 4635 μg/kg of DON for phase 3. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CPK, creatine phosphokinase. [a,b] Means with different superscripts differ ($p < 0.05$). [X,Y] Means with different superscripts tend to differ ($0.05 \leq p < 0.10$).

There were no differences for protein carbonyl, malondialdehyde, total glutathione, tumor necrosis factor-alpha (TNF-α), immunoglobulin A (IgA), immunoglobulin G (IgG), or interleukin 8 (IL-8) in jejunal mucosa among pigs from experimental groups (Table 3).

Table 3. Immune and oxidative stress markers from gut mucosa in newly-weaned pigs fed diets high (HM) or low (LM) in mycotoxins [1] or postbiotic yeast cell wall-based blend (PYCW) for 36 days.

Diet	LM		HM		SEM	*p* Value		
PYCW	0%	0.2%	0%	0.2%		Diet	PYCW	Diet vs. PYCW
Concentration/mg of protein								
Protein carbonyl, mMol	2.41	2.26	1.97	2.14	0.30	0.206	0.999	0.416
Malondialdehyde, µM	0.382	0.288	0.349	0.350	0.105	0.845	0.536	0.523
Total glutathione, µM	3.02	4.45	4.58	3.74	1.49	0.691	0.784	0.291
TNF-α, pg	4.53	3.86	4.45	5.49	1.19	0.501	0.870	0.458
TNF-α/IgA	3.20	2.32	3.77	2.50	0.93	0.534	0.084	0.753
IgA, µg	2.44	2.15	1.45	2.87	0.74	0.804	0.281	0.108
IgG, µg	1.36	1.48	1.25	1.46	0.31	0.815	0.540	0.885
IL-8, ng	0.255	0.215	0.207	0.270	0.071	0.949	0.825	0.299

[1] HM has 1.0 µg/kg of AFB1 and 2258 µg/kg of DON on average for phases 1 and 2, and 44.6 µg/kg of AFB1 and 4635 µg/kg of DON for phase 3. TNF-α, tumor necrosis factor-alpha; IL-8, interleukin 8; IgA, immunoglobulin A; IgG, immunoglobulin G.

There were significant interactions for apparent ileal digestibility of dry matter ($p < 0.05$), gross energy ($p < 0.05$), and nitrogen ($p < 0.05$) among pigs from experimental groups (Table 4). These interactions indicate that when there is no PYCW, HM reduced ($p < 0.05$) the apparent ileal digestibility of dry matter, gross energy, and nitrogen. The interactions also indicate that within pigs fed LM, the addition of PYCW decreased ($p < 0.05$) the apparent ileal digestibility of dry matter, gross energy, and nitrogen. Feeding HM also decreased ($p < 0.05$) the apparent ileal digestibility of ether extract.

Table 4. Apparent ileal digestibility of dry matter, gross energy, nitrogen, and ether extract in diets high (HM) or low (LM) in mycotoxins [1] or postbiotic yeast cell wall-based blend (PYCW) fed to newly-weaned pigs for 36 days.

Diet	LM		HM		SEM	*p* Value		
PYCW	**0%**	**0.2%**	**0%**	**0.2%**		**Diet**	**PYCW**	**Diet vs. PYCW**
Dry matter, %	66.5^{ax}	55.9^{y}	53.4^{b}	53.7	2.2	0.001	0.023	0.018
Gross energy, %	70.0^{ax}	59.4^{y}	56.6^{b}	56.6	2.0	<0.001	0.011	0.011
Nitrogen, %	80.4^{ax}	74.9^{y}	72.6^{b}	74.1	1.1	<0.001	0.065	0.002
Ether extract, %	98.2	97.6	97.1	96.7	0.4	0.009	0.179	0.888

[1] HM has 1.0 µg/kg of AFB1 and 2258 µg/kg of DON on average for phases 1 and 2, and 44.6 µg/kg of AFB1 and 4635 µg/kg of DON for phase 3. [a,b] Means with different superscripts differ ($p < 0.05$). [x,y] Means with different superscripts differ ($p < 0.05$).

In addition, feeding HM decreased ($p < 0.05$) crypt depth from mid jejunum, but no other differences were observed for intestinal histomorphology on villus width, villus height to crypt depth ratio, nor on proportion of Ki-67-positive cells (Table 5).

Table 5. Intestinal morphology and Ki-67 [1] proportion in histology sections of mid jejunum in newly-weaned pigs fed diets high (HM) or low (LM) in mycotoxins [2] or postbiotic yeast cell wall-based blend (PYCW) for 36 days.

Diet	LM		HM		SEM	*p* Value		
PYCW	**0%**	**0.2%**	**0%**	**0.2%**		**Diet**	**PYCW**	**Diet vs. PYCW**
Villus width, µm	420.9	432.4	401.9	407.7	7.1	0.263	0.684	0.611
Villus height (V), µm	153.2	157.7	161.2	160.7	19.8	0.100	0.512	0.826
Crypt depth (C), µm	214.0	223.4	203.4	203.5	11.6	0.045	0.522	0.527
V:C	2.00	1.97	2.02	2.04	0.14	0.651	0.932	0.785
Ki-67 proportion, %	25.93	25.61	27.27	27.44	2.40	0.343	0.963	0.882

[1] Ki-67 is an estimate of the proliferative rate, calculated based on the proportion of cells positive to Ki-67 staining (immunohistochemistry) to the total number of cells in the crypt. [2] HM has 1.0 µg/kg of AFB1 and 2258 µg/kg of DON on average for phases 1 and 2, and 44.6 µg/kg of AFB1 and 4635 µg/kg of DON for phase 3.

2.4. Discussion

Considering that naturally contaminated feedstuffs were used for diet formulation, a myriad of mycotoxins were detected, suggesting contamination of multiple fungi. Mycotoxin-contaminated corn was added only to the phase 3 diet to model a real scenario in a commercial farm, where pigs may be fed diets with higher mycotoxin levels as they get older. Such a scenario may happen as older pigs are less susceptible to mycotoxins [27] and, thus, feedstuffs potentially contaminated with mycotoxins may be fed to older pigs. The overall concentration of mycotoxins was three-fold higher in HM than in LM diets, aiming to exceed the guidance levels of mycotoxins' (AFB1 and DON) concentration in the United States (20 and 1000 µg/kg, [11,13]) and Europe (10 and 900 µg/kg, [12,14]) and cause detrimental effects by feeding HM diet to pigs. The advisory levels of DON for swine are 5000 µg/kg in grain, with grain feedstuffs not to exceed 20% of the final diet [28]. This would result in a concentration of 1000 µg/kg of the final diet. As intended in the current study, aflatoxins and DON concentrations in HM surpassed the values preconized for feeding nursery pigs in the United States. In phases 1 and 2, both LM and HM diets were below (1.0 µg/kg) the threshold for AFB1, whereas in phase 3, HM diet was above the threshold (44.6 µg/kg). For DON in phases 1 and 2, HM diet was above (2258 µg/kg) the threshold for the mycotoxin. During phase 3, both LM and HM diets were above (1311 vs. 4635 µg/kg) the threshold for DON. A lower percentage of DON contaminated DDGS was used on phase 3 (20.8%) in comparison to phases 1 and 2 (22%). However, the supplemental mycotoxin (difference between LM and HM) was achieved as intended (supplemental 2000 µg of DON per kg of feed) in the current study (supplemental 2163 µg of DON per kg of feed).

The levels of DON in the current study are considered as the sum of DON and its acetylated and glycosylated forms. Even though the DON-3-glucoside does not seem to have

detrimental effects [29] this choice was based on the previously proven ability of the gut microbiome to convert DON-3-glucoside into the toxic form (DON) both *in vitro* [30] and *in vivo* [31] in pigs. Regarding the acetylated forms, they may show either less, similar, or more adverse effects than DON [29,32]. Comparable to DON-3-glucoside, 3-acetyl-DON and 15-acetyl-DON can be converted to DON and, thus, were considered together to have similar effects as DON [33]. Nivalenol is also a type B trichothecene, but its conversion to DON has not been documented. Besides, nivalenol presents stronger harmful effects than DON [32,34] and, thus, is considered as a distinct mycotoxin in the current as well as in other scientific publications [35].

Zearalenone average concentration in LM and HM diets (44.3 and 96.0 µg/kg of feed) did not surpass the guidance level of 100 µg/kg preconized in Europe [14] for young pigs. Indeed, previous studies show that zearalenone in this range would not affect health and growth of pigs at 7 to 30 kg body weight [35–38]. There are no regulations concerning zearalenone concentration in swine feed in the United States. The sum of fumonisins B1, B2, and B3 observed in the current study did not surpass the recommended maximum level set by the Food and Drug Administration [13] of 10,000 µg/kg for pigs. Similarly, the sum of fumonisins B1 and B2 detected in diets was below the guidance level of 5000 µg/kg recommended for pigs in Europe [14]. Considering the low level of contamination of zearalenone and fumonisins, their impacts on health and growth of pigs in this study would be insignificant and, therefore, are not discussed further.

In the present study, it was observed that feeding HM (aflatoxins at 1.0 µg/kg of feed and DON at 2094 µg/kg of feed) caused reduced feed intake in newly-weaned pigs during phase 1. For phases 2 and 3, AFB1 (1.0 and 44.6 µg/kg) and DON (2421 and 4635 µg/kg) levels were higher and reduced feed intake. In high and acute doses, deoxynivalenol is known to cause

vomiting and impair feed intake [36]. In low and chronic doses, deoxynivalenol depresses feed intake, especially in pigs, due to reduced peristalsis mediated by local serotonin [37,38] and satiety signaling mediated by peptide YY (peptide tyrosine tyrosine) [39] along with the release of pro-inflammatory cytokines [40,41]. Aflatoxins also modulate cytokine expression by reducing IL-1β and increasing IL-10 [42] and TNF-α when in combination with DON [7]. In a study performed with mice, DON depressed feed intake as early as 2 h after the ingestion in a dose-response manner [39]. The mechanism underlying feed refusal is related to the increase in peptide YY and serotonin plasma levels, leading to satiety perception, as shown in a previous study [43]. The lower energy and nutrient intake, negatively impacted by HM after phase 1, led to impaired animal growth during the entire period, as observed in ADG. Our study is in accordance with results obtained by Chaytor et al. [7], where AFB1 and DON could impair animal growth at 60 and 300 µg/kg of feed, respectively. Current results are supported by our outcomes observed for gross energy and nitrogen apparent ileal digestibility, where feeding HM reduced the digestibility when no PYCW was added. It was previously shown that DON at 10,000 µg/kg of feed is able to reduce digestibility of essential amino acids in pigs [44]. Similarly, the current study illustrates that, by the end of phase three, pigs fed HM (DON at 4635 µg/kg and AFB1 at 44.6 µg/kg of feed) showed impaired energy and nitrogen apparent ileal digestibility in comparison to pigs fed LM (DON at 1311 µg/kg of feed).

Comparing growth performance for phases 1, 2 and 3, feeding HM reduced ADG by 22, 15, and 14%, and ADFI was reduced by 22, 15 and 12%, respectively. This result suggests that HM effects were stronger during phase 1, while animals were recovering from weaning stress [45]. The interaction in ADG during phase 1 shows that feeding diets with mycotoxins decreased pig ADG when the PYCW was not added to diets. Of interest, when PYCW was added, feeding

HM did not reduce ADG during phase 1. At the same time, the interaction in G/F shows that pigs fed HM had greater G/F when PYCW was included. Indeed, PYCW tended to improve G/F among pigs fed MT, indicating that PYCW provided improved growth performance in challenged pigs. During the first week of phase 2, animals fed HM showed greater G/F probably due to its lower ADFI (on average 116 g lower). This is a result of an evolutional adaptation, where pigs eating reduced amounts of energy and nutrient have a compensatory improvement in feed efficiency [46]. In the overall period, adding PYCW tended to reduce G/F, and such decrease in efficiency was supported by the reduction of gross energy and nitrogen apparent ileal digestibility when adding PYCW to LM.

It was not possible to distinguish if the inclusion of AFB1-contaminated corn in phase 3 diet solely influenced the variables tested or if AFB1 inclusion showed additive or synergistic activity that could eventually potentialize one or other deleterious effects of mycotoxins. Comparing growth performance for phase 2 and phase 3, the difference between animals fed LM and animals fed HM was similar for ADG (15 and 14%), but the difference widens for ADFI (15 and 12%), and G/F (−1% and 3%). The observed outcomes are likely to be related to characteristics of each group in the beginning of phase 3, instead of AFB1 addition. The lower body weight of pigs from the mycotoxin group, respectively 9 and 11% lower for phase 2 and phase 3, may have influenced such results, as animals with a smaller body size need less energy and nutrients for maintenance and, thus, can direct these for tissue deposition [47].

Besides the aforementioned effects on pig growth, DON can debilitate liver and kidney function [48,49]. In addition, AFB1 has also shown deleterious effects upon liver and mineral balance in pigs [50]. Weaver et al. [8] has revealed that the combination of AFB1 and DON (AFB1 at 150 and DON at 1100 μg/kg of feed) caused liver damage. In the current study,

feeding HM reduced albumin and total protein concentration in serum, which may indicate that liver protein synthesis was compromised due to mycotoxin toxicity [51]. Aflatoxins are known to impair cell protein synthesis through inhibition of RNA polymerase activity in the nucleus [52], resulting in reduced cell viability [53]. Taken together, aflatoxin effects could be responsible for the hypoproteinemia observed as well as impaired pig growth. Mycotoxins tended to decrease the ALT, a cytosolic enzyme investigated to assess liver and kidney functions [54]. Even though an increase in ALT would be expected, cases of chronic liver damage are associated with the reduction in serum levels of the enzyme [55], as observed in the current study. The assumption of liver damage is supported by the tendency to decreased concentrations of cholesterol in pigs fed diets with mycotoxins, as cholesterol is mainly synthetized by hepatocytes [56]. An increase in creatinine level in serum is observed in the case of liver failure [57,58], but, unexpectedly, a decrease in serum creatinine in pigs fed diets with mycotoxins was observed in the current study. Creatinine level in serum linearly increases with pig body weight [59], thus, this could be the reason for the higher creatinine level observed in the group fed LM, which was 3.36 kg heavier at d 36 than pigs fed HM. However, considering there were no changes in BUN, BUN/creatinine, or alkaline phosphatase ratio in animals consuming mycotoxins, it is possible to infer that liver function was not greatly affected in the impairment of nitrogen excretion [60–62]. The CPK increase in serum can be indicative of severe hepatic [63] or muscular damage [64]. Hence, the tendency for decreased CPK, and reduced AST and AST/ALT promoted by PYCW suggest that the addition of PYCW may have induced a protective effect in the liver and muscle, reducing the release of their enzymes in serum. The relative level between AST and ALT can be a more reliable variable to evaluate chronic liver damage in humans [65]. Such correlation indicating liver damage can also be observed in pigs challenged with bacterial toxin [66] and under

mycotoxin challenge [67]. Furthermore, stimulation of protein synthesis and proliferation in muscular cells proportioned by n-butyric acid [68], one of the components in PYCW, could have mitigated the muscular damage caused by mycotoxins.

The reduction in BUN/creatinine along with the increase in glucose suggest that animals fed PYCW could more efficiently utilize protein and carbohydrate sources in feed. Indeed, yeast cell wall supplementation above 0.05% has been shown to play a role in modulating amino acid and glucose levels in blood serum of pigs [69]. Mycotoxins decreased phosphorus levels in serum of animals in the absence of PYCW, but the addition of PYCW mitigated the deleterious effects of mycotoxins on phosphorus levels in pigs fed HM. Mycotoxin damage to kidney and liver [48,70] may have caused the alteration in phosphorus levels, considering that the liver is the main site for cholesterol synthesis, vitamin D precursor, and that both organs are essential for vitamin D activation [71,72]. Reinforcing this line of thought, and as aforementioned, mycotoxins in fact decreased cholesterol level in serum of pigs fed diets with mycotoxins. In addition, previous data have shown toxic effects of aflatoxin on kidney, calcium and vitamin D metabolism in broilers [73]. The PYCW was able to mitigate mycotoxic effects on phosphorus balance, as seen by the tendency towards increased phosphorus levels in serum of pigs fed diets with mycotoxins. However, further investigation into vitamin D levels as well as kidney and liver function would be necessary to determine if the decrease in phosphorus was related to vitamin D metabolism observed in the current study. Of interest, one of the components of PYCW is vitamin C. The vitamin C pool can be depleted under challenging situations as it is involved in the reduction of oxidative stress [74]. Furthermore, vitamin C supplementation in weaned pigs is related to improved immune function [75].

Pigs challenged with DON have shown an increase in inflammatory cytokine expression as IL-8, as well as up-regulation of glutathione peroxidase 2 gene and IgG [76]. In the present study, impacts of mycotoxins were not strong enough to affect oxidative damage markers, pro-inflammatory cytokines, or immunoglobulins in the jejunal mucosa. In a previous study conducted by Pasternak et al. [77], where pigs chronically exposed to DON (3800 µg/kg of feed vs. 4635 µg/kg of feed in the current study), IL-8 in the ileum was the only cytokine upon which effects of mycotoxin presented a trend to increase. Thus, the relative low mycotoxin level used seemed unlikely to promote changes in immunological variables.

It was possible to observe that ileal apparent digestibility for gross energy and nitrogen were reduced when adding PYCW to LM. Yeast cell wall is mostly composed of β-D-glucans and mannose-oligosaccharides; the latter has been shown to improve nutrient digestibility when supplemented in a concentrated form from 0.1 to 0.2% in diets [78] with the effect being more pronounced during the first two weeks after weaning [79]. The β-glucans have shown to improve nutrient digestibility in weaned pigs when treated with antibiotics [80]. In the current study, however, there was a lack of difference in nutrient digestibility when adding PYCW in pigs fed HM. Alternatively, the current reduction in apparent ileal digestibility of nutrients in feed observed in pigs fed LM with PYCW could be due to reduced digestibility of β-D-glucans derived from yeast non-starch polysaccharides [81].

The decrease in crypt depth was the only noticeable effect in animals consuming mycotoxins. DON might have impaired cell proliferation in the crypts by inhibiting the Wnt/β-catenin pathway [82]. Nivalenol has shown greater impact in jejunal morphology than DON [34] and the ileum is the most affected segment of the small intestine regarding protein synthesis [76,83], which may explain why no major effects on gut morphology were detected.

Chronic mycotoxin challenge with DON (3050 µg/kg, for 7 to 25 kg body weight) and AFB1 (44.6 µg/kg, for 16 to 25 kg body weight) clearly impaired growth performance, reduced apparent ileal digestibility of nutrients in feeds, and caused mild liver damage in newly-weaned pigs. The postbiotic yeast cell wall-based blend partly reduced liver damage. In pigs not challenged with mycotoxins, the postbiotic yeast cell wall-based blend reduced apparent ileal digestibility of dry matter, gross energy, and nitrogen; whereas in pigs challenged with mycotoxins, the postbiotic yeast cell wall-based blend maintained growth performance and apparent ileal digestibility of all nutrients in feeds.

2.5. Conclusion

Chronic dietary challenge of DON (3050 µg/kg) and AFB1 (44.6 µg/kg) is harmful to newly-weaned pigs, compromising growth and nutrient digestibility. Supplementation with the postbiotic yeast cell wall-based blend could partially overcome the harmful effects of the dietary challenge of multiple mycotoxins on growth and health of weanling pigs.

2.6. Materials and methods

The Institutional Animal Care and Use Committee (IACUC) at North Carolina State University (Raleigh, NC, USA) reviewed and approved the protocol of this experiment.

2.6.1. Animals and Experimental Diets

The levels of selected mycotoxins detected in conventional corn DDGS, DON-contaminated DDGS, and aflatoxin-contaminated corn used for diet formulation are presented on Table 6.

Table 6. Selected mycotoxins detected in conventional dried distillers grains with solubles (DDGS), deoxynivalenol (DON) contaminated DDGS, and aflatoxin (AF) contaminated corn used for diet formulation to newly-weaned pigs for 36 d.

Mycotoxin, µg/kg	Conventional DDGS	DON DDGS	AF Corn
Aflatoxin B1	0.1	0.1	239.6
Aflatoxin B2	0.5	0.5	30.9
Aflatoxin G1	0.1	0.1	14.8
Aflatoxin G2	0.1	0.1	0.1
Deoxynivalenol	2064	5897	4
3-acetyl-deoxynivalenol	21.7	102.9	2.3
15-acetyl-deoxynivalenol	550	2104	2
Deoxynivalenol-3-glucoside	38.7	11.0	11.0
Nivalenol	49.9	49.9	49.9
Fusarenon-X	2.5	2.5	2.5
Fumonisin B1	479	347	29,773
Fumonisin B2	27	37	5478
Fumonisin B3	5	17	6092
Zearalenone	213	1720	3

Mycotoxin concentrations were measured at the Analytical Services Laboratory of Alltech Inc. Laboratory (37+™, Alltech Inc., Nicholasville, KY, USA). The detection limit was used for variables not detected. Levels of mycotoxins reported considered values above the limit of quantitation of each mycotoxin, the relative standard deviation (< 20%), and the signal to noise ratio (> 10).

Experimental diets were formulated to meet or exceed the nutrient requirements suggested by the National Research Council (NRC) [84] following a three-phase feeding program (Table 7). The use of three dietary phases followed the recommendation of the NRC to meet nutritional requirements of nursery pigs [84]. All experimental diets were sampled (from nine different locations, 2 kg total per diet), with 200 g of each being sent to North Carolina Department of Agriculture (Raleigh, NC, USA) and to the Analytical Services Laboratory of Alltech Inc. (37+™, ISO/IEC 17025:2005 official accreditation (No. 79481) using LC-MS/MS; Alltech Inc., Nicholasville, KY, USA) for analyses of nutrient composition and mycotoxin concentration, respectively (Table 8). The sample processing for quantitative determination of mycotoxin concentration followed the procedures previously described by Jackson et al. [85]. In short, ground and homogenized feed samples had 400 mg subsampled and placed in silanized

glass vials for extraction with acetonitrile/water/formic acid (84.0:15.9:0.1, *v*/*v*/*v*) during 18 h. Vials were centrifuged and the supernatant was dried at room temperature for 30 min under nitrogen stream. A mixture of water/acetonitrile/formic acid (95.0:4.9:0.1, v/v/v) with 10 mmol/L of ammonium acetate was used as loading buffer for analysis of 44 mycotoxins by LC-MS/MS.

Table 7. Composition of experimental diets high (HM) or low (LM) in mycotoxins in a three-phase feeding program fed to newly-weaned pig for 36 d [1].

Item	Phase1 (d 0 to 7)		Phase2 (d 7 to 21)		Phase3 (d 21 to 36)	
	LM	HM	LM	HM	LM	HM
Ingredient, %						
Ground corn	14.67	14.67	31.07	31.07	45.80	41.05
Aflatoxin corn[2]	-	-	-	-	-	4.75
Corn DDGS	22.00	-	22.00	-	20.81	-
DON corn DDGS[3]	-	22.00	-	22.00	-	20.81
Soybean meal	16.00	16.00	19.00	19.00	28.36	28.36
Whey permeate	20.00	20.00	10.00	10.00	-	-
Cookie meal	10.00	10.00	5.00	5.00	-	-
Poultry meal	6.00	6.00	4.00	4.00	-	-
Blood plasma	5.00	5.00	3.00	3.00	-	-
Fish meal	2.00	2.00	-	-	-	-
Poultry fat	2.00	2.00	3.00	3.00	1.90	1.90
Limestone	0.90	0.90	1.05	1.05	1.09	1.09
Dicalcium phosphate	-	-	0.50	0.50	0.76	0.76
Salt	0.22	0.22	0.22	0.22	0.21	0.21
L-lysine HCl	0.53	0.53	0.51	0.51	0.35	0.35
DL-methionine	0.15	0.15	0.12	0.12	0.04	0.04
L-threonine	0.10	0.10	0.10	0.10	0.03	0.03
Mineral mix	0.15	0.15	0.15	0.15	0.14	0.14
Vitamin mix	0.03	0.03	0.03	0.03	0.03	0.03
Zinc oxide	0.25	0.25	0.25	0.25	-	-
Titanium dioxide	-	-	-	-	0.48	0.48
Calculated composition						
DM, %	91.10	91.10	90.48	90.48	89.57	89.57
ME, kcal/kg	3471	3471	3480	3480	3391	3391
SID Lys, %	1.50	1.50	1.35	1.35	1.23	1.23
SID Thr, %	0.88	0.88	0.80	0.80	0.73	0.73
SID Trp, %	0.25	0.25	0.22	0.22	0.23	0.23
SID Met + Cys, %	0.82	0.82	0.74	0.74	0.68	0.68
Ca, %	0.85	0.85	0.80	0.80	0.71	0.71
STTD P, %	0.47	0.47	0.41	0.41	0.33	0.33
DON[4], µg/kg	0	2000	0	2000	0	2000
AF, µg/kg	0	0	0	0	0	200

[1] Postbiotic yeast cell wall-based blend (PYCW) was added to LM and HM at 0.2% in all phases to create two other dietary treatments. [2] Aflatoxin corn, aflatoxin-contaminated corn (mycotoxin concentration: 285 µg/kg of aflatoxins and 41.3 mg/kg of feed of fumonisins). [3] Deoxynivalenol (DON) corn dried distillers grains with solubles (DDGS), deoxynivalenol-contaminated corn DDGS (mycotoxin concentration: 8115 µg/kg of feed of deoxynivalenol and 401 µg/kg of feed of fumonisins). [4] Deoxynivalenol concentration is reported as sum of deoxynivalenol and its metabolites: 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, and deoxynivalenol-3-glucoside. Ingredients in bold were included or not (no inclusion is represented by a dash "-") depending on the dietary treatment. DM, dry matter; ME, metabolizable energy; SID, standard ileal digestibility; and STTD, standard total tract digestibility.

Table 8. Selected mycotoxins detected in diets high (HM) or low (LM) in mycotoxins in a 3-phase feeding program fed to newly-weaned pig for 36 d.

Mycotoxin, µg/kg	Phase / Diet [1]	1		2		3	
		LM	HM	LM	HM	LM	HM
Aflatoxin B1		0.1	0.1	0.1	0.1	0.1	43.7
Aflatoxin B2		0.5	0.5	0.5	0.5	0.5	0.5
Aflatoxin G1		0.1	0.1	0.1	0.1	0.1	0.1
Aflatoxin G2		0.1	0.1	0.1	0.1	0.1	0.1
Deoxynivalenol		501	1534	524	1837	1050	3956
3-acetyl-deoxynivalenol		2.3	28.6	2.3	29.7	2.3	25.2
15-acetyl-deoxynivalenol		145	521	154	543	106	435
Deoxynivalenol-3-glucoside		11	11	11	11	152	220
Nivalenol		49.9	49.9	49.9	49.9	49.9	49.9
Fusarenon-X		2.5	2.5	2.5	2.5	2.5	2.5
Fumonisin B1		130	156	219	239	53	1,289
Fumonisin B2		40.9	41.7	39.1	19.0	1.8	67.5
Fumonisin B3		5.0	5.0	5.0	5.0	5.0	41.3
Zearalenone		3	3	128	242	3	43

Mycotoxin concentrations were measured at the Analytical Services Laboratory of Alltech Inc. Laboratory (37+™, Alltech Inc., Nicholasville, KY, USA). The detection limit was used for variables not detected. Levels of mycotoxins reported considered values above the limit of quantitation of each mycotoxin, the relative standard deviation (< 20%), and the signal to noise ratio (> 10). [1] Postbiotic yeast cell wall-based blend (PYCW) was added to LM and HM at 0.2% in all phases to create two other dietary treatments at each phase.

Forty-eight (24 barrows and 24 gilts) crossbred pigs (PIC 337 × Camborough 22) were weaned at 21 d of age (7.49 ± 0.11 kg). Subsequent to weaning, pigs were moved to the research farm and allotted to four dietary treatments based on a completely randomized block design according to sex and body weight (heavy, medium, and light) with two factors for 36 days based on a three-phase feeding program. Pigs within the heavy body weight group ranged from 9.12 to 7.68 kg, within the medium body weight group the range was 7.64 to 7.28 kg, and within the light body weight group the range was 7.2 to 6.04 kg. The two factors were: (1) dietary mycotoxins, obtained from naturally contaminated DDGS (DON: 887.3 or 3050 µg/kg of feed during all phases) and corn (AFB1: 1.0 or 44.6 µg/kg of feed during phase 3), and (2) PYCW (0 or 0.2%; Alltech Inc., Nicholasville, KY, USA). The PYCW is a proprietary blend of postbiotic functional bioactive constituents containing hydrolyzed yeast cell wall of *Saccharomyces*

cerevisiae, organic acids (n-butyric acid), vitamins (ascorbic acid), and essential oils (rosemary extract, Alltech Inc., Nicholasville, KY, USA). An overview of pig assignment to treatments according to the factors and mycotoxin levels per treatment is detailed in Figure 1.

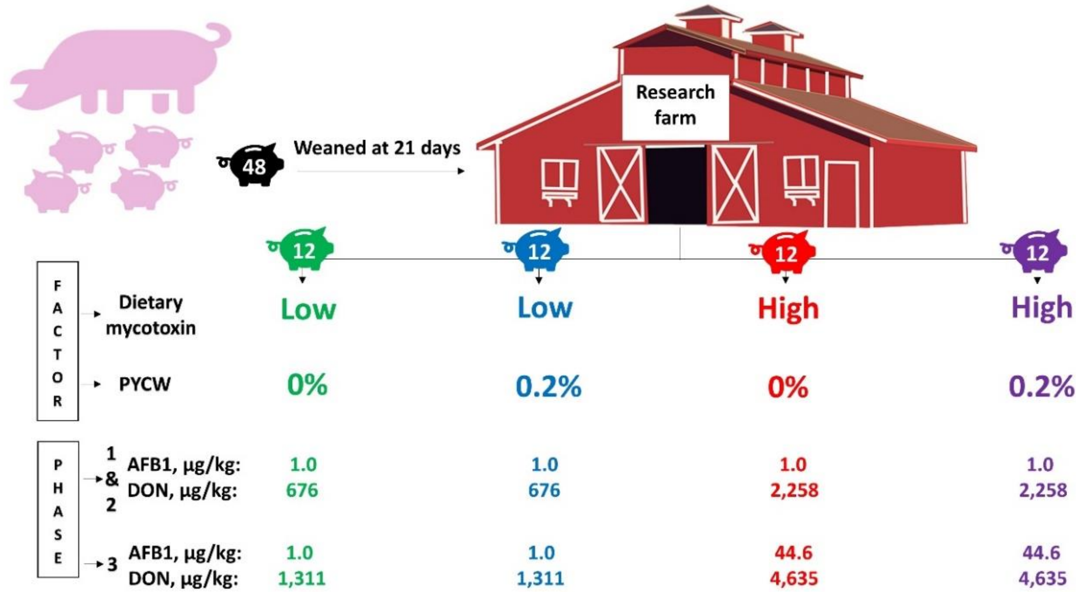

Figure 1. Experimental arrangement and pig assignment to treatments. Forty-eight pigs were weaned at 21 days of age and assigned to four dietary treatments (n = 12) following a randomized complete block design in a 2 × 2 factorial arrangement. Pigs in green received low mycotoxin diet (LM; formulated without the dietary mycotoxin factor—conventional DDGS and corn); pigs in blue received LM and postbiotic yeast cell wall-based blend (PYCW) at 0.2%; pigs in red received high mycotoxin diet (HM; formulated with dietary mycotoxin factor—DON-contaminated DDGS and, during phase 3, aflatoxin-contaminated corn); and pigs in purple received HM and PYCW at 0.2%. The average aflatoxin B1 (AFB1) and deoxynivalenol (DON) levels during phases 1 and 2, and during phase 3 are specified per treatment.

2.6.2. Data Collection

Body weight of pigs and feed consumption by pigs were recorded weekly and used to obtain average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G/F). On d 35, 10 mL blood samples from the external jugular vein were collected with 0.8 × 32 mm needles (Eclipse, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and serum

blood collection tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) using a vacutainer tube holder, following procedures routinely employed by our research group [20,86,87]. Serum samples were stored at −80 ˚C in a freezer (812660-760, Thermo Fisher Scientific, Waltham, MA, USA) in 1.5 mL tubes (Fisherbrand, Fisher Scientific, Hampton, NH, USA) after centrifugation at 1509× *g* at 4 ˚C for 15 min (5811F, Eppendorf, Hamburg, HH, Germany). Serum samples were used for measuring serum biochemistry (including serum proteins, enzymes, cholesterol, blood urea nitrogen, creatinine, and glucose), and electrolyte profiling (Antech Diagnostics Laboratory, Cary, NC, USA).

At the end of the study (d 36), pigs were euthanized to obtain scrapped mucosa and intact tissue from mid-jejunum and ileal digesta. Pigs were stunned by captive bolt followed by vena cava exsanguination. Samples of gut mucosa (from 15 cm) from the mid-jejunum were scraped with the aid of clean histological slides. The mid-jejunum of pigs was determined at 3.5 m distal of the duodenum [88]. Mucosal samples were frozen in liquid nitrogen immediately after collection and then transferred to −80 ◦C until laboratory analyses. Ileal digesta samples were obtained by gently squeezing from the ileocecal junction until the proximal end of the ileocecal fold. Ileal digesta containers were emerged in ice and then stored at −80 ◦C until laboratory analyses. Tissue samples (5 cm) from the mid-jejunum were placed in 10% buffered formaldehyde at room temperature until further processing for histological evaluation.

2.6.3. Laboratory Analyses

For protein extraction mid-jejunum mucosa samples were thawed on ice and 1 g of the sample was placed in sterile tube (5 mL tube, Eppendorf, Hamburg, Germany) followed by addition of 2 mL of PBS (MP Biomedicals, Inc., Santa Ana, CA, USA). Samples were

homogenized (Tissuemiser, Thermo Fisher Scientific, Waltham, MA, USA) for 30 s and centrifuged at 87,000× g for 20 min on ice. The supernatant was subdivided into vials stored at −80 ◦C until being used to evaluate antioxidant status, immune response, and intestinal barrier function in mid-jejunum mucosa relative to the protein content of samples (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Protein quantification started by mixing 25 µL of each sample with 200 µL of working reagent provided in the kit in a microplate well (96-Well EIA/RIA Plates, Corning, Corning, NY, USA), followed by 30 s incubation in plate shaker. The plate was covered with clear adhesive strip and incubated for 30 min at 37 ◦C. The plate was cooled to room temperature and wells were read at 562 nm.

The quantification of protein carbonyls (STA-310, Cell Biolabs, Inc., San Diego, CA, USA) as an index of oxidized proteins is described by Shen et al. [89]. Briefly, the protein content of each sample determined in the previous assay was diluted to 10 µg/mL. Diluted samples (100 µL) were pipetted into wells and incubated for 2 h at 37 ◦C. Each well was washed three times with 250 µL of PBS (MP Biomedicals, Inc., Santa Ana, CA, USA) and 100 µL of working solution supplied in the kit added before plate incubation in the dark for 45 min. Each well was washed with 250 µL of PBS/ethanol (1:1, v/v) and incubated for 5 min in an orbital shaker; this procedure was repeated four times. Each well was washed with 250 µL of PBS twice, 200 µL of blocking solution was added, and the plate was incubated for 1 h in an orbital shaker. Each well was washed with 250 µL of washing buffer three times and 100 µL of anti-dinitrophenylhydrazine antibody supplied in the kit were added according to dilutions recommended by the manufacturer. The plate was incubated in an orbital shaker for 1 h. Each well was washed with 250 µL of washing buffer three times and 100 µL of horseradish peroxidase antibody were added for incubation for 1 h in an orbital shaker. Each well was

washed with 250 μL of washing buffer five times, 100 μL of substrate were added, and 100 μL of stop solution were added after the onset color development. The wells were read at 450 nm.

Malondialdehydes (STA-330, Cell Biolabs, Inc., San Diego, CA, USA) were measured by incubating for 5 min 100 μL of each sample in equal volume of SDS lysis solution provided in the kit. Followed by incubation at 95 ◦C for 45 min with 250 μL of the reagent (130 mg of thiobarbituric acid in 25 mL of diluent) supplied in the kit, which had the pH adjusted (Accumet AB15 pH Meter, Fisher Scientific, Hampton, NH, USA) to 3.5 with sodium hydroxide. Tubes were cooled in for 5 min and centrifuged at 4000× g for 15 min. The supernatant (300 μL) was vigorously mixed with 300 μL of butanol for 2 min and centrifuged at 10,000× g for 5 min. The supernatant (200 μL) was transferred to a microplate (96-Well EIA/RIA Plates, Corning, Corning, NY, USA) and samples were read at 532 nm.

Tumor necrosis factor-α (PTA00, R&D Systems, Inc., Minneapolis, MN, USA) was measured by pipetting 50 μL of assay diluent supplied in the kit with 50 μL of samples into wells. The plate was covered with clear adhesive strip and incubated for 2 h. Each well was washed five times with 300 μL of washing buffer, 100 μL of TNF-α conjugate supplied in the kit were added, and the plate was incubated following same specifications. Each well was washed five times with 300 μL of washing buffer, 100 μL of substrate solution supplied in the kit were added to each well, and the plate was incubated for 30 min in the dark. After incubation, 100 μL of stop solution supplied in the kit were added and wells were read 450 and 570 nm to obtain reading at 570 subtracted from 450 nm.

Iterleukin-8 quantification (P8000, R&D Systems, Inc., Minneapolis, MN, USA) was performed by pipetting 50 μL of assay diluent supplied in the kit with 100 μL of samples into wells. The plate was covered with clear adhesive strip and incubated for 2 h in orbital shaker at

500 rpm. Each well was washed five times with 300 μL of washing buffer, 200 μL of porcine IL-8 conjugate supplied in the kit were added, and the plate was incubated following same specifications. Each well was washed five times with 300 μL of washing buffer, 120 μL of substrate solution supplied in the kit were added, and the plate incubated for 30 min in the dark. After incubation, 120 μL of stop solution supplied in the kit were added and wells were read 450 and 570 nm to obtain reading at 570 subtracted from 450 nm.

Immunoglobulin A (E100-102, Bethyl Laboratories, Inc., Montgomery, TX, USA) and IgG (E100-104, Bethyl Laboratories, Inc., Montgomery, TX, USA) were measured by pipetting 100 μL of their respective affinity purified antibody in each well according to the kit dilution. The plate was incubated for 1 h. Each well was washed five times with 260 μL of washing buffer supplied in the kit, 200 μL of blocking buffer supplied in the kit were added, and the plate was incubated for 30 min. Each well was washed five times with 260 μL of washing buffer, 100 μL of samples were added and incubated for 30 min. Each well was washed five times with 260 μL of washing buffer, 100 μL of diluted horseradish peroxidase supplied in the kit were added, and the plate was incubated for 1 h. Each well was washed five times with 260 μL of washing buffer, 100 μL of tetramethylbenzidine substrate were added, and the plate was incubated in the dark for 15 min. Sulfuric acid (100 μL) at 0.18 M was used as stop solution. The plate was read at 450 nm.

For measurement of total glutathione, a different protein extraction method was used, as recommended by the kit manufacturer total glutathione (STA-312, Cell Biolabs, Inc., San Diego, CA, USA). Mid-jejunum mucosa (100 mg) and 1 mL of metaphosphoric acid at 5% were mixed and homogenized with a glass pestle. The homogenate was centrifuged at 64,000× g for 15 min. The supernatant was used for total glutathione determination total glutathione (STA-312, Cell

Biolabs, Inc., San Diego, CA, USA). Glutathione reductase solution (25 µL), NADPH solution (25 µL) supplied in the kit, and samples (100 µL) were added to each well. The chromogen solution (100 µL) supplied in the kit was added to each well and the plate was read at 405 nm every 2 min during 10 min.

All wavelengths (for quantifications of protein, protein carbonyls, malondialdehydes, total glutathione, TNF-α, IL-8, IgA, and IgG) were read at the same microplate reader (Synergy HT, Biotek, Winooski, VT, USA).

Ileal digesta was freeze dried (SP Scientific, Virtis 24DX48 GPFD/300820, Warminster, PA, USA) and ground. Subsamples of ground material were analyzed for apparent ileal digestibility of dry matter [90], gross energy (6200 Calorimeter, Parr Instrument Company, Moline, IL, USA), nitrogen (method 990.03, [91], ATC Scientific, North Little Rock, AR, USA), and ether extract (method 920.39, [91]).

Fixed mid-jejunal tissue was removed from 10% buffered formaldehyde after two weeks for the obtainment of two transversal cuts that were transferred histological cassettes and submerged in 70% ethanol. Mid-jejunal cuts were included in paraffin for assembling histological slides after staining for Ki-67 antigen. The immunohistochemistry staining with Ki-67 primary monoclonal antibody (1:500 dilution) followed by anti-mouse secondary antibody (1:2 dilution factor) and the use of diamino-benzamine reagent for color development was performed in accordance with methods previously described by Kim et. al. [20]. Ten pictures of each pig were used to measure gut morphology by a single researcher choosing a well-oriented villus and its associated crypt. Measurements included: villus width (at half of villus height), villus height (from tip of the villus to top of the crypt), crypt depth (from top to bottom of the crypt), and calculating villus height: crypt depth [86]. The proportion of proliferating cells in the

crypt was also estimated by calculating the proportion of cells positive to Ki-67 after taking pictures at 40× in Sony Van–Ox S microscope (Opelco, Washington, DC, USA) and processing in ImageJS tool [92] for analysis as described by Holanda and Kim [86].

2.6.4. Data Analyses and Interpretation

The statistical analysis was performed using the mixed procedure of SAS 9.3 software (Cary, NC, USA). Factors, dietary mycotoxins and PYCW, and interaction were considered as main effects, whereas blocks, sex and initial body weight, were considered as random effects. Means were obtained by the LSMEANS statement. In case of interaction, treatments were compared with the PDIFF statement and tested by Tukey test. Results were considered statistically different for $p < 0.05$ and were considered as tendency for $0.05 \leq p < 0.10$.

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CHAPTER 3. EFFICACY OF MYCOTOXIN DETOXIFIERS ON HEALTH AND GROWTH OF NEWLY-WEANED PIGS UNDER CHRONIC DIETARY CHALLENGE OF DEOXYNIVALENOL

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3.1. Abstract

The efficacy of yeast-based mycotoxin detoxifiers on health and growth performance of newly-weaned pigs (27-d-old) fed diets naturally contaminated with deoxynivalenol was investigated. Sixty pigs were individually assigned to five treatments for 34 d: NC (negative control, 1.2 mg/kg of deoxynivalenol); PC (positive control, 3.2 mg/kg of deoxynivalenol); CYC (PC + clay/yeast culture-based product, 0.2%); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants-based product, 0.2%); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants-based product, 0.2%). Blood and jejunal mucosa were sampled, and data were analyzed using Proc Mixed of SAS with pre-planned contrasts. Deoxynivalenol reduced the average daily gain (ADG) in phase 3. Pigs fed CYC had greater overall ADG, average daily feed intake during phase 3, and gain to feed ratio during phase 2 than PC. At d 14, deoxynivalenol reduced blood urea nitrogen/creatinine and tended to reduce blood urea nitrogen. Pigs fed CYB tended to have greater aspartate aminotransferase than PC. At d 34, pigs fed CYC and CYB tended to have lower serum creatine phosphokinase than PC. Pigs fed CYE had lower blood urea nitrogen/creatinine than PC. In jejunal mucosa, deoxynivalenol tended to increase malondialdehydes and decrease glutathione. Pigs fed CYE and CYB had lower malondialdehydes, pigs fed CYB had greater glutathione and tended to have lower immunoglobulin A than PC. Pigs fed CYC and CYE tended to have lower interleukin 8 than PC. In summary, deoxynivalenol challenge (1.2 vs. 3.2 mg/kg) mildly compromised growth performance and increased the oxidative stress of pigs. Mycotoxin detoxifiers could partially overcome deoxynivalenol toxicity enhancing liver health, whereas CYE and CYB reduced oxidative stress, and CYC and CYB reduced immune activation. In conclusion, yeast-based detoxifiers with functional components as clay/inactivated yeast/botanicals/antioxidants had

increased detoxifying properties in newly-weaned pigs challenged with deoxynivalenol, potentially by enhancing adsorbability, immune function, gut health, and reducing oxidative stress.

Keywords: deoxynivalenol; health; yeast

3.2. Introduction

Mycotoxins are fungal metabolites that may have deleterious effects in animals [1]. Deoxynivalenol is a type B trichothecene produced by *Fusarium* species that may contaminate cereal grains used to formulate diets for livestock animals. Mycotoxins are detected in cereal grains worldwide, with a prevalence of 88% on feed and raw feedstuffs [2]. *Fusarium* toxins are the most prevalent globally, as well as in the United States, showing a higher frequency in corn and wheat and also occurring in byproducts of the food chain as bread/cookie meal, dried distillers grains with solubles (DDGS), and brewery wastes [2–5]. Corn is one of the main feedstuffs used in pig diets, where type B trichothecenes had on average 78% of occurrence and 1.235 mg/kg in samples from the United States in three years (2016–2018) [3]. Pigs are the most susceptible species to deoxynivalenol among domestic animals, in which deoxynivalenol can reduce feed intake, impair animal growth, trigger pro-inflammatory, and immunological responses, and even cause vomiting when in high concentrations in diets [6–10]. At the cellular level, deoxynivalenol induces a ribotoxic stress response (p38 mitogen-activated protein kinase, MAPK, activation by phosphorylation), regulating gene expression, by inhibiting translation, and triggering apoptosis, by the inhibition of protein elongation and the activation of apoptosis factors (nuclear factor-κB) [11,12]. Deoxynivalenol can also naturally occur as deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, deoxynivalenol-3-sulfate, and deoxynivalenol-15-sulfate [13,14]. As extensively reviewed, although they are less toxic than deoxynivalenol, the deoxynivalenol modified forms can be converted to deoxynivalenol and then absorbed in the gastrointestinal tract of the pig, except for deoxynivalenol sulfates [12,13,15,16]. Thus, the modified forms may potentiate deoxynivalenol toxicity when present in animal feed. The co-contamination with other mycotoxins may influence deoxynivalenol kinetics and

toxicity. For instance, another *Fusarium* toxin named culmorin can hinder the deoxynivalenol detoxification step of glucuronidation [17]. Therefore, the Food and Drug Administration stipulated advisory levels for not exceeding 1 mg/kg in diets for growing pigs [18]. Similarly, the deoxynivalenol limit in Europe for pigs is 0.9 mg/kg of feed [19]. Collectively, the high prevalence of deoxynivalenol, its toxicity for pigs, and the existing advisory levels make investigations for an effective mycotoxin deactivator valuable.

The use of mycotoxin detoxifiers as feed additives aims to reduce mycotoxin toxicity in contaminated feed ingredients, enabling their use for animal feed formulation [20]. There is a wide array of mycotoxin detoxifiers, with an equivalent myriad of claims. Mycotoxin detoxifiers may directly interact with deoxynivalenol molecules by the chemical transformation of deoxynivalenol to non-toxic compounds, or by the adsorbent properties of inorganic (clays) or organic substances (algal extracts, yeast, and yeast byproducts). However, it has been a great challenge to find interacting substances that can effectively counteract deoxynivalenol toxicity, because of the small chemical structure and low polarity of deoxynivalenol [10,21,22].

Mycotoxin detoxifiers that target deoxynivalenol often include components to promote gut health, stimulate the immune system, or provide sources of functional and conditionally essential nutrients(dietary fiber, plant derivatives, and nucleotides), combining different properties and improving detoxification ability [23]. Therefore, three yeast-based mycotoxin detoxifiers were chosen for evaluations under deoxynivalenol challenge in nursery pigs. First, CYC product (clay and yeast culture) is composed of bentonite clay, yeast culture (*Saccharomyces cerevisiae*, minimum 2.7×10^{10} CFU/kg), diatomaceous earth, and dehydrated kelp meal. Second, CYE product (clay, yeast, and plant extract) is composed of organo-aluminosilicate clays, a blend of hydrated sodium-calcium aluminosilicate clays, yeast cell walls,

plant extracts, and antioxidants. The third, CYB product (clay, yeast, and botanicals) is composed of high adsorbent modified clay minerals, inactivated yeast, and fermentation extracts (*S. cerevisiae*), antioxidant and preservative mixture (calcium propionate), and botanicals (milk thistle, rosemary, licorice, and boldo).

Deoxynivalenol contamination in feedstuffs may range from 0 to 50 mg/kg, but it is most commonly lower than 5 mg/kg [24]. Specifically for naturally contaminated diets, the same study highlights that reductions in pig feed intake and growth are observed with more than 1 to 2 mg/kg, where an additional 1 mg/kg of deoxynivalenol results in an 8% decrease in pig growth [24]. Based on such outcomes, average levels in similar studies [6,8,10,25,26], and the advisory levels in the United States, the current study used diets naturally contaminated with an additional 2 mg/kg of deoxynivalenol (1.2 vs. 3.2 mg/kg). The first objective was to evaluate deoxynivalenol effects on health and growth performance in nursery pigs, due to the species susceptibility to deoxynivalenol, particularly at an early age. Second, to test the effects and efficacy of three yeast-based mycotoxin detoxifiers on reducing deoxynivalenol toxicity in pigs.

3.3. Results

The average high and low temperatures were 30.4 and 27.5 ◦C, with 74.6% specific humidity over the course of the whole experiment. There were no differences in the body weight of pigs among treatments during the whole experiment (Figure 1). Pigs fed PC (positive control, 3.2 mg/kg of deoxynivalenol) presented a tendency for lower average daily gain (ADG) from d 21 to 28 ($p = 0.058$) and d 28 to 34 ($p = 0.087$), and significantly lower ($p < 0.05$) during the whole phase 3 (d 21 to 34), in comparison to pigs fed NC (negative control, 1.2 mg/kg of deoxynivalenol; Table 1). Pigs fed PC presented a tendency ($p = 0.099$) for lower average daily

feed intake (ADFI) from d 21 to 28, in comparison to pigs fed NC. Pigs fed PC showed a tendency for lower gain to feed ratio (G:F) during phase 2 (d 7 to 21; $p = 0.066$) and from d 14 to 21 ($p = 0.087$) in comparison to pigs fed NC. Pigs fed CYC (PC + CYC product at 0.2%) had greater ($p < 0.05$) ADG during phase 3 (d 21 to 34) and from d 28 to 34, than pigs fed PC. Pigs fed CYE (PC + CYE product at 0.2%) showed a higher fecal score than pigs fed PC on d 5 (Figure 2). There were no differences in the fecal score among pigs from experimental treatments on d 7 or 14.

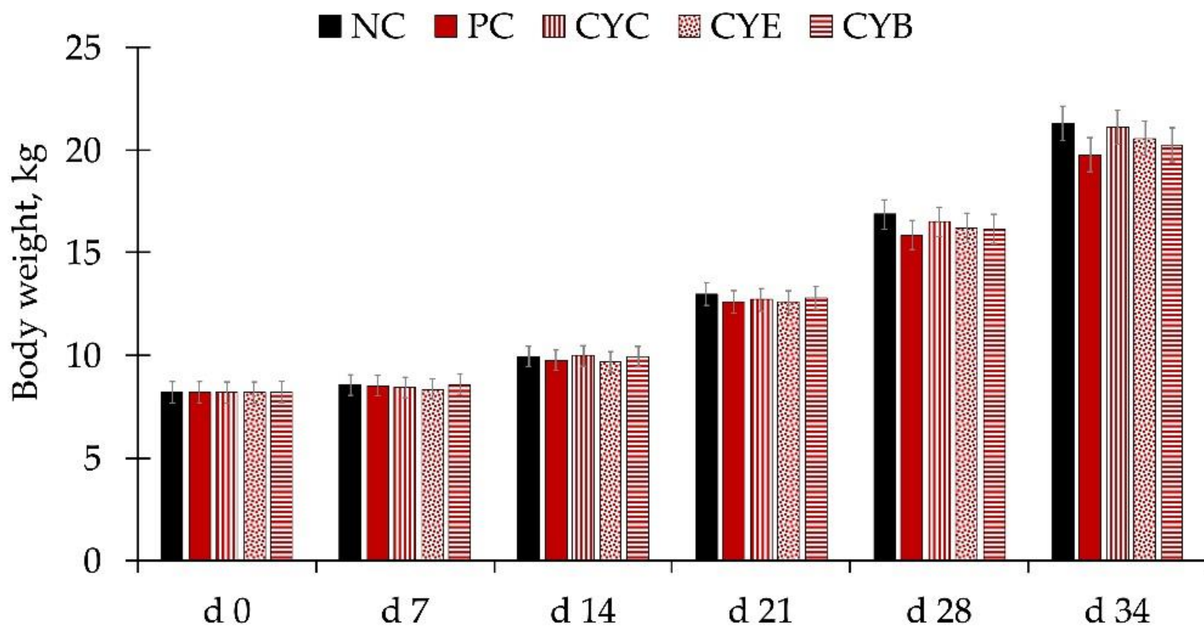

Figure 1. Body weight in newly-weaned pigs consuming diets with (PC, positive control diet with 3.2 mg/kg of deoxynivalenol) or without deoxynivalenol (NC, negative control diet with 1.2 mg/kg of deoxynivalenol) and diets with deoxynivalenol and mycotoxin detoxifiers at 0.2%: CYC (PC + clay/yeast culture-based product); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants-based product); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants-based product).

Table 1. Animal performance variables in newly-weaned pigs consuming diets with (PC [1]) or without deoxynivalenol (NC) and diets with deoxynivalenol and mycotoxin detoxifiers [2].

Treatment	NC	PC	Additive			SEM	p Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
ADG[3], g/d										
Phase 1 (d 0 to 7)	49	47	35	23	52	26	0.939	0.668	0.399	0.868
Phase 2 (d 7 to 21)	316	291	303	303	301	25	0.416	0.705	0.708	0.752
d 7 to 14	199	176	218	191	191	26	0.479	0.205	0.648	0.632
d 14 to 21	434	407	389	415	411	30	0.493	0.651	0.835	0.920
Phase 3 (d 21 to 34)	641	551	649	615	573	31	0.033	0.024	0.124	0.592
d 21 to 28	555	465	544	517	480	34	0.058	0.102	0.267	0.743
d 28 to 34	740	651	771	729	681	40	0.087	0.026	0.132	0.561
Overall (d 0 to 34)	385	340	380	365	354	22	0.106	0.162	0.377	0.627
ADFI[4], kg/d										
Phase 1 (d 0 to 7)	115	124	114	112	100	21	0.649	0.602	0.525	0.223
Phase 2 (d 7 to 21)	394	391	371	388	387	31	0.951	0.559	0.915	0.892
d 7 to 14	261	246	252	243	248	29	0.592	0.828	0.937	0.942
d 14 to 21	526	537	491	532	526	39	0.809	0.321	0.913	0.805
Phase 3 (d 21 to 34)	954	852	940	904	857	50	0.168	0.161	0.396	0.930
d 21 to 28	800	702	788	769	732	46	0.099	0.157	0.253	0.613
d 28 to 34	1,096	1,027	1,118	1,060	1,004	61	0.374	0.253	0.664	0.768
Overall (d 0 to 34)	544	512	536	528	508	34	0.393	0.531	0.667	0.897
G:F[5]										
Phase 1 (d 0 to 7)	0.35	0.14	0.27	0.31	0.25	0.22	0.460	0.656	0.746	0.688
Phase 2 (d 7 to 21)	0.81	0.74	0.82	0.79	0.77	0.03	0.066	0.045	0.217	0.399
d 7 to 14	0.76	0.72	0.88	0.68	0.76	0.08	0.749	0.178	0.674	0.742
d 14 to 21	0.84	0.75	0.80	0.80	0.78	0.03	0.087	0.329	0.310	0.615
Phase 3 (d 21 to 34)	0.68	0.66	0.69	0.68	0.67	0.03	0.406	0.372	0.261	0.749
d 21 to 28	0.70	0.67	0.69	0.68	0.65	0.03	0.425	0.544	0.622	0.717
d 28 to 34	0.68	0.66	0.69	0.70	0.68	0.04	0.603	0.463	0.302	0.537

Table 2. (Continued).

Treatment	NC	PC	Additive			SEM	*p* Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
Overall (d 0 to 34)	0.71	0.67	0.71	0.70	0.69	0.02	0.112	0.145	0.236	0.330

[1] PC diets have 2 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS; [2] Mycotoxin detoxifiers were added to PC at 0.2% in all phases to create three other dietary treatments: CYC (PC + clay/yeast culture based product); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants based product); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants based product); [3] ADG, average daily gain; [4] ADFI, average daily feed intake; [5] G:F, gain to feed ratio.

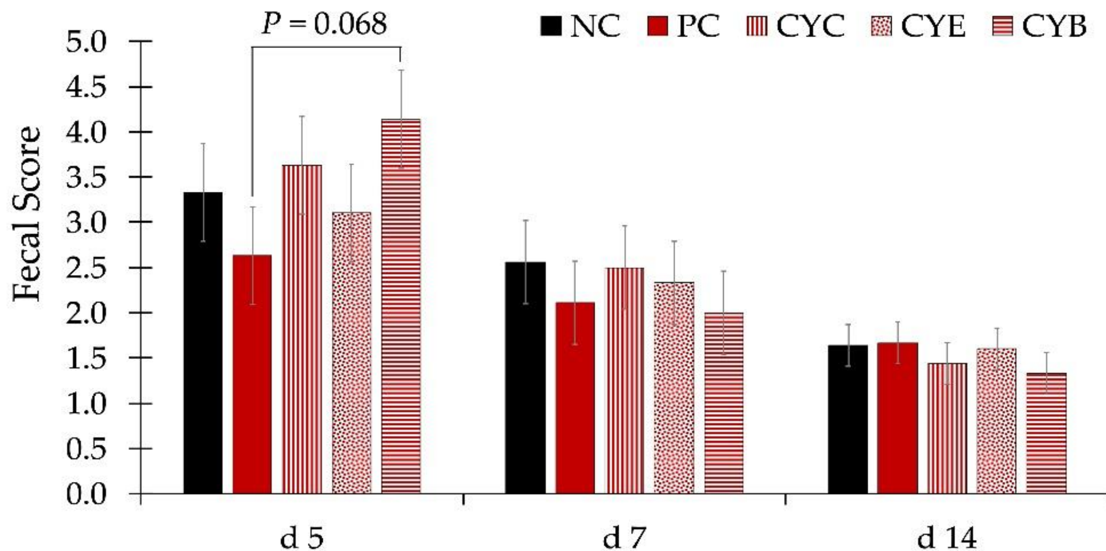

Figure 2. Fecal score in newly-weaned pigs consuming diets with (PC, positive control diet with 3.2 mg/kg of deoxynivalenol) or without deoxynivalenol (NC, negative control diet with 1.2 mg/kg of deoxynivalenol) and diets with deoxynivalenol and mycotoxin detoxifiers at 0.2%: CYC (PC + clay/yeast culture-based product); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants-based product); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants-based product). The fecal score was subjectively measured by a single evaluator based in a 1 to 5 scale, as described by Hu et al. [27].

Regarding the assessment of proteins, metabolites, and electrolytes in blood serum on d 14, pigs fed PC showed a tendency to lower ($p = 0.078$) blood urea nitrogen levels and lower ($p < 0.05$) blood urea nitrogen to creatinine ratio than pigs fed NC (Table 2). Pigs fed CYC showed a tendency ($p = 0.056$) for lower total protein and lower ($p < 0.05$) albumin levels than pigs fed

PC. Pigs fed CYE showed a tendency for lower albumin ($p = 0.086$) and albumin to globulin ratio ($p = 0.063$) than pigs fed PC. Pigs fed CYB (PC + CYB product at 0.2%) had lower ($p < 0.05$) albumin, higher ($p < 0.05$) globulin, and lower ($p < 0.05$) albumin to globulin ratio than pigs fed PC. Pigs fed CYB showed a tendency ($p = 0.086$) for higher aspartate aminotransferase than pigs fed PC. Pigs fed CYB had a lower ($p < 0.05$) calcium concentration than pigs fed PC.

Table 3. Serum variables for proteins, metabolites, and electrolytes in newly-weaned pigs consuming diets with (PC) or without deoxynivalenol (NC) and diets with deoxynivalenol and mycotoxin detoxifiers on d 14.

Treatment	NC	PC	Additive			SEM	*p* Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
Total protein, g/dL	4.65	4.66	4.45	4.62	4.54	0.11	0.937	0.056	0.695	0.275
Albumin, g/dL	3.05	3.16	2.90	2.98	2.85	0.07	0.283	0.015	0.086	0.003
Globulin, g/dL	1.60	1.50	1.55	1.63	1.69	0.10	0.265	0.600	0.139	0.036
Albumin/globulin	1.98	2.15	1.91	1.87	1.74	0.13	0.268	0.115	0.063	0.008
AST[1], IU/L	47.08	36.50	46.00	43.00	50.25	5.18	0.147	0.202	0.370	0.062
ALT[2], IU/L	17.92	17.08	16.90	17.58	16.83	0.99	0.461	0.876	0.658	0.825
ALP[3], IU/L	222.6	205.7	231.0	216.3	205.7	17.8	0.416	0.235	0.607	1.000
BUN[4], mg/dL	14.25	12.25	11.69	10.50	12.17	0.97	0.078	0.621	0.121	0.941
Creatinine, mg/dL	0.93	0.94	0.89	0.86	0.88	0.07	0.761	0.389	0.132	0.289
BUN/creatinine	15.83	13.17	13.43	12.42	14.00	1.29	0.031	0.829	0.535	0.491
Glucose, mg/dL	79.83	79.58	82.08	75.33	78.83	6.65	0.968	0.697	0.498	0.905
Cholesterol, mg/dL	66.50	72.08	72.35	79.25	71.08	6.71	0.447	0.972	0.330	0.891
CPK[5], IU/L	2982	1598	2361	1590	1607	670	0.147	0.430	0.993	0.992
Phosphorus, mg/dL	9.26	9.32	9.69	9.53	9.73	0.44	0.870	0.311	0.543	0.254
Calcium, mg/dL	9.73	9.82	9.55	9.63	9.39	0.24	0.678	0.192	0.341	0.038
Sodium, mEq/L	143.9	143.7	144.5	143.7	144.0	1.2	0.835	0.511	1.000	0.782
Potassium, mEq/L	6.19	6.19	6.29	6.35	6.21	0.24	1.000	0.744	0.640	0.961
Sodium/potassium	23.75	23.58	23.27	22.92	23.67	0.86	0.891	0.803	0.584	0.945
Chloride, mEq/L	105.4	106.3	106.4	105.3	105.9	0.8	0.461	0.921	0.376	0.767

[1] AST, aspartate amino transferase; [2] ALT, alanine amino transferase; [3] ALP, alkaline phosphatase; [4] BUN, blood urea nitrogen; [5] CPK, creatine phospho-kinase.

On d 34, a blood serum assessment of proteins, metabolites, and electrolytes showed that pigs fed CYC had higher glucose and tended to show lower creatine phosphokinase than pigs fed PC (Table 3). Pigs fed CYE had higher ($p < 0.05$) blood urea nitrogen to creatinine ratio than

pigs fed PC. Pigs fed CYB showed a tendency for lower creatine phosphokinase ($p = 0.071$) and calcium ($p = 0.051$) than pigs fed PC.

Table 4. Serum variables for proteins, metabolites, and electrolytes in newly-weaned pigs consuming diets with (PC) or without deoxynivalenol (NC) and diets with deoxynivalenol and mycotoxin detoxifiers on d 34.

Treatment	NC	PC	Additive			SEM	*p* Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
Total protein, g/dL	4.88	4.78	4.58	4.77	4.66	0.10	0.462	0.109	0.892	0.312
Albumin, g/dL	3.11	3.06	2.91	3.03	2.86	0.09	0.674	0.188	0.821	0.075
Globulin, g/dL	1.77	1.73	1.67	1.73	1.80	0.07	0.604	0.571	0.929	0.424
Albumin/globulin	1.76	1.80	1.80	1.78	1.63	0.09	0.778	1.000	0.895	0.190
AST[1], IU/L	38.18	41.00	35.45	37.92	33.42	4.55	0.665	0.395	0.628	0.236
ALT[2], IU/L	20.82	22.83	21.09	21.92	20.58	1.57	0.364	0.435	0.673	0.303
ALP[3], IU/L	244.4	237.4	265.2	253.8	240.2	16.6	0.775	0.203	0.439	0.897
BUN[4], mg/dL	13.6	12.5	11.7	13.4	12.5	1.2	0.288	0.455	0.411	0.388
Creatinine, mg/dL	0.77	0.74	0.71	0.67	0.71	0.04	0.548	0.505	0.138	1.000
BUN/creatinine	17.6	17.1	16.3	21.4	17.9	1.57	0.758	0.695	0.028	0.506
Glucose, mg/dL	102.6	93.3	107.6	100.2	99.7	4.2	0.124	0.020	0.246	0.665
Cholesterol, mg/dL	64.91	65.00	65.16	66.42	65.08	3.80	0.981	0.973	0.763	0.282
CPK[5], IU/L	3003	4244	1564	2156	1477	1083	0.423	0.087	0.170	0.071
Phosphorus, mg/dL	11.10	10.58	10.58	10.96	10.82	0.37	0.182	0.986	0.299	0.517
Calcium, mg/dL	9.84	9.89	9.91	9.93	9.53	0.13	0.763	0.925	0.853	0.051
Sodium, mEq/L	139.4	137.8	138.5	139.1	138.0	0.8	0.139	0.495	0.213	0.867
Potassium, mEq/L	5.56	5.40	5.54	5.62	5.58	0.19	0.544	0.613	0.412	0.507
Sodium/potassium	25.64	25.75	25.18	24.92	25.00	0.79	0.920	0.614	0.450	0.497
Chloride, mEq/L	100.5	99.0	100.3	99.8	99.3	0.7	0.109	0.148	0.345	0.776

[1] AST, aspartate amino transferase; [2] ALT, alanine amino transferase; [3] ALP, alkaline phosphatase; [4] BUN, blood urea nitrogen; [5] CPK, creatine phospho-kinase.

Pigs fed PC tended to have a higher concentration of malondialdehydes ($p = 0.069$) and to have lower total glutathione ($p = 0.067$) in mid-jejunal mucosa, in comparison to pigs fed NC (Table 4). Pigs fed CYC tended to present lower ($p = 0.079$) interleukin 8 than pigs fed PC. Pigs fed CYE had lower ($p < 0.05$) malondialdehydes than pigs fed PC. Pigs fed CYB had lower ($p < 0.05$) malondialdehydes and higher ($p < 0.05$) glutathione than pigs fed PC. Pigs fed CYB tended to have lower ($p = 0.081$) immunoglobulin A than pigs fed PC. There were no differences in

protein carbonyls, tumor necrosis factor-alpha, or immunoglobulin G in the mid-jejunal mucosa of pigs among experimental treatments.

Table 5. Inflammatory response and oxidative stress markers quantification from mid-jejunal mucosa in newly-weaned pigs consuming diets with (PC) or without deoxynivalenol (NC) and diets with deoxynivalenol and mycotoxin detoxifiers on d 34.

Treatment	NC	PC	Additive			SEM	*p* Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
Concentration/mg of protein										
Protein carbonyl, nmol	3.608	3.190	4.343	2.770	3.886	0.748	0.660	0.238	0.659	0.464
Malondialdehyde, μM	0.404	0.619	0.452	0.305	0.371	0.108	0.069	0.164	0.009	0.037
Total glutathione, μM	4.274	2.594	2.424	3.232	4.439	0.641	0.067	0.854	0.481	0.045
TNF-α[1], pg	1.091	1.196	0.859	1.031	1.190	0.243	0.759	0.356	0.630	0.984
IL-8[2], ng	0.507	0.661	0.468	0.486	0.513	0.076	0.150	0.079	0.102	0.166
IgA[3], μg	2.610	4.032	3.528	2.752	2.284	0.734	0.154	0.618	0.198	0.081
IgG[4], μg	2.169	1.657	1.710	1.811	2.144	0.309	0.242	0.905	0.722	0.266

[1] TNF-α, tumor necrosis factor alpha; [2] IL-8, interleukin 8; [3] IgA, immunoglobulin A; [4] IgG, immunoglobulin G.

There were no differences in apparent ileal digestibility for dry matter, gross energy, nitrogen, and ether extract (Table 5), or for morphology or estimated proliferative rate of enterocytes in the crypt after Ki-67 staining performed in histology sections from the mid-jejunum (Figure 3) of pigs among experimental treatments.

Table 6. Apparent ileal digestibility in newly-weaned pigs consuming diets with (PC) or without deoxynivalenol (NC) and diets with deoxynivalenol and mycotoxin detoxifiers on d 34.

Treatment	NC	PC	Additive			SEM	*p* Value			
			CYC	CYE	CYB		NC vs. PC	PC vs. CYC	PC vs. CYE	PC vs. CYB
Dry matter, %	61.92	53.31	43.86	51.61	55.95	6.87	0.189	0.160	0.794	0.608
Gross energy, %	59.67	52.07	51.77	57.27	56.59	6.25	0.248	0.965	0.429	0.491
Nitrogen, %	70.80	64.76	67.51	69.62	67.34	5.42	0.203	0.569	0.305	0.584
Ether extract, %	96.64	95.79	95.74	96.50	96.21	0.76	0.372	0.961	0.456	0.635

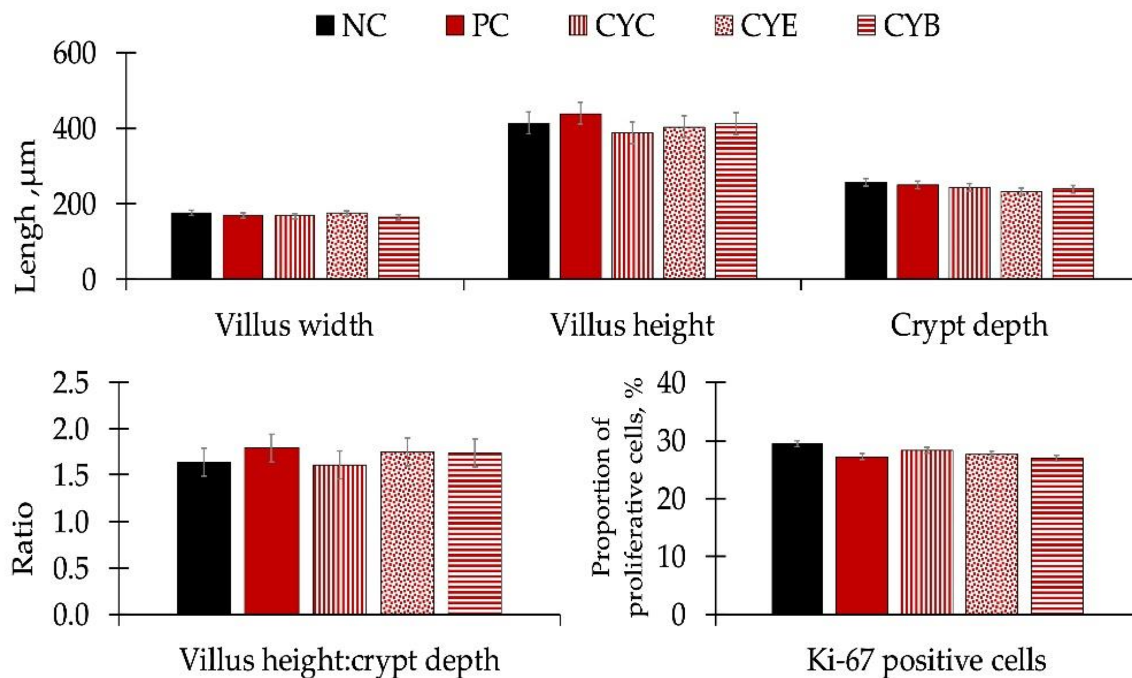

Figure 3. Intestinal morphology (top and bottom left graphs) and proportion of proliferative cells (bottom right) in the mid-jejunum sections of newly-weaned pigs consuming diets with (PC, positive control diet with 3.2 mg/kg of deoxynivalenol) or without deoxynivalenol (NC, negative control diet with 1.2 mg/kg of deoxynivalenol) and diets with deoxynivalenol and mycotoxin detoxifiers at 0.2%: CYC (PC + clay/yeast culture-based product); CYE (PC + clay/yeast cell wall/plant extracts/antioxidants-based product); and CYB (PC + clay/inactivated yeast/botanicals/antioxidants-based product) on d 34.

3.4. Discussion

The use of mycotoxin detoxifiers with multiple compositions has shown promising effects over mycotoxin detoxifiers with single components, notably when animals are challenged with deoxynivalenol [23]. Therefore, we tested three mycotoxin detoxifiers (CYC, CYE, and CYB) with multiple components in the current study. The chosen mycotoxin detoxifiers have in common variable sources of yeast. The CYC supplement is composed of bentonite, yeast culture (*S. cerevisiae*, minimum 2.7×10^{10} CFU/kg), diatomaceous earth, and dehydrated kelp meal. Bentonites have a weak binding ability *in vitro* for deoxynivalenol (3.24%), when compared to other mycotoxins, such as aflatoxins (92.5%) [28]. The lower adsorbability for deoxynivalenol in

comparison to aflatoxins is probably due to the latter's higher polarity [22,29]. However, *in vivo*, bentonites have shown the ability to reduce deoxynivalenol's detrimental effect on bone mineralization in minks fed naturally contaminated diets with the mycotoxin [30]. The inclusion of yeast culture could potentiate bentonites effects by fermentation of sugars in the gastrointestinal tract yielding metabolites, with beneficial effects on the nutrition and health of pigs, like peptides and organic acids [31]. Indeed, yeast culture supplementation has shown improved total tract apparent digestibility, reduced *Escherichia coli* count in feces, and enhanced animal health, improving animal performance [32]. Diatomaceous earth has an intermediary binding ability to mycotoxins according to the review prepared by Huwig et al. [29], but its adsorbability is relatively high among mineral sources and it is among the ones with the highest adsorbability considering *Fusarium* toxins. The last component of CYC, kelp meal, may present antioxidant properties, being able to improve growth performance in broilers challenged with mycotoxins [33], as well as in pigs challenged with *E. coli* [34].

The CYE supplement is composed of organo-aluminosilicate clays and a blend of hydrated sodium-calcium aluminosilicate clays (sepiolite), yeast cell walls (from *S. cerevisiae*), plant extracts (a mixture of aromatic substances), triglycerides, calcium propionate, and antioxidants (citric acid, BHT, and ethoxyquin). The aluminosilicates have a relatively low adsorbing capacity to mycotoxins, but their adsorbability is greatly improved when in the hydrated sodium-calcium form, especially concerning aflatoxins [29], or when aluminosilicates are bound to organic components [35]. Yeast cell wall components, such as β-D-glucans and glucomannans, have *Fusarium* toxin adsorbing ability [36]. Indeed, processing yeast to yield yeast cell walls may enhance the binding ability to mycotoxins [29]. In porcine intestinal cells challenged with deoxynivalenol, the yeast cell wall could enhance enterocyte integrity and

reduce inflammation [23]. *In vivo* studies with yeast cell wall as a unique additive to mitigate deoxynivalenol toxicity in pigs are scarce. The scarcity of publications is probably due to the limited effect of the yeast cell wall on improving animal health and growth in pigs challenged with deoxynivalenol [37,38], playing a minor role as a deoxynivalenol detoxifier. The plant extracts and antioxidants were included in this blend to promote gut health, by reducing the oxidative stress that may be provoked by mycotoxin challenge.

The CYB supplement is composed of high adsorbent modified clay minerals (sepiolite and bentonite), inactivated yeast and fermentation extracts (from *S. cerevisiae*), antioxidant (propyl gallate) and preservative mixture (calcium propionate extract), and botanicals (milk thistle seed, *Silybum marianum*; rosemary dried leaves, *Rosmarinus officinalis* L.; licorice root; and boldo dried leaves, *Peumus boldus*). These components have similar properties as the ones described for the first two mycotoxin detoxifiers. The yeast component in CYB can be roughly classified as an intermediary between CYC and CYE. The fermentation extracts may have similar beneficial effects of the yeast metabolites in CYC, but they will, rather, be present in the additive instead of being produced in the gut, because of using inactivated yeast. At the same time, the use of inactivated yeast may pose a role similar to CYE by enabling yeast cell wall interaction with enterocytes and with deoxynivalenol, enhancing gut health and acting as a mycotoxin detoxifier, respectively. The distinct component, in comparison to previous mycotoxin detoxifiers, is calcium propionate. Calcium propionate, as an organic acid, can lower the pH in the gastrointestinal tract, promoting gut health and nutrient digestibility [39]. Furthermore, calcium propionate is known to hinder fungal development in feed [40]. Regarding the mycotoxin challenge, calcium propionate could decrease mycotoxin accumulation in body tissues, improve liver health, and promote recovery in growth performance as shown in a study

conducted with broilers [41]. In pigs, a mycotoxin detoxifier similar to CYB was tested against zearalenone, aflatoxin, and ochratoxin, showing promising detoxifying effects by improving gut health, nutrient digestibility and absorption, and animal growth [42].

In the current study, it was possible to notice the chronic toxicity of deoxynivalenol in pigs, after feeding deoxynivalenol contaminated diet for 21 d. When comparing pigs fed PC and NC, signals of deoxynivalenol impairment on growth performance started to be noticed after phase 2. For ADFI and ADG, deoxynivalenol toxicity was noticeable during phase 3, reducing both variables after d 21, when comparing pigs fed PC and NC. The higher fecal score observed for pigs fed CYE in comparison to pigs fed PC may suggest that pigs fed diets with CYE were more susceptible to deoxynivalenol toxicity in the gastrointestinal tract, resulting in a higher incidence of soft feces. The use of yeast cell walls, instead of the whole cell used in CYC and CYB, may have accounted for the increase in fecal score observed for CYE, but not to CYC and CYB. Ultimately, pigs fed CYC, showed greater G:F during phase 2 and ADG during phase 3 than pigs fed PC. This may suggest that the use of whole yeast cells or their fermentation products (CYC and CYB) may have induced gut health. Reinforcing such finding, pigs facing weaning stress supplemented with yeast culture showed increased IFN-γ production, suggesting cellular immunity recruitment in the gut, and eventual reduced lymphocyte T CD4+ activation [31,32]. Thus, the supposed increase in cellular immunity could have supported gut health post-weaning in CYC and CYB in the current study.

Feeding a deoxynivalenol contaminated diet with 3.2 mg/kg of feed did not show major impacts on growth performance in weaned pigs during 34 d. Deoxynivalenol is a mycotoxin known to depress animal feed intake [43] and may cause vomiting [7], resulting in impaired animal growth [44]. Indeed, in the review prepared by Pestka [11], reduced feed intake and

growth are characteristics of deoxynivalenol chronic exposure, as observed in the current study, due to the upregulation of proinflammatory cytokines by p38 MAPK induced activation. Whereas vomiting is observed under acute exposure mediated by the increase in serotonin and peptide YY, where the latter also plays a role in appetite inhibition [7,43]. Considering that our research group has been performing related investigations, the number of replications and the mycotoxin concentration were expected to result in impaired animal growth [8,25,26,45,46]. Thus, it can be hypothesized that deoxynivalenol concentration in the NC diet (1 mg/kg of feed), could have impaired growth performance of pigs and was masking the outcomes observed in comparison to pigs fed PC. Supporting this hypothesis, it was observed in previous studies that deoxynivalenol concentrations of 0.6 mg/kg in naturally contaminated feed can impair the growth performance of pigs [8,24]. Thus, deoxynivalenol toxicity in PC treatment in comparison to NC was not able to elicit acute and marked differences in the growth performance of pigs.

Unexpectedly, pigs fed PC had lower blood urea nitrogen and blood urea nitrogen to creatinine ratio than pigs fed NC on d 14. Nitrogen concentration in blood and its ratio to creatinine are related to liver and kidney health since both organs are the major sites of amino acid deamination, urea synthesis, and excretion. Pigs fed CYC presented lower total protein and albumin levels than pigs fed PC. Albumin is the major constituent of serum proteins; thus, its decrease may have led to the decrease observed in total protein. Similarly, pigs fed CYE and CYB presented lower albumin levels as well as for the ratio of albumin to globulin than pigs fed PC. The interaction of deoxynivalenol with the 60S ribosomal subunit hinders protein translation, also known as ribotoxic stress [12], which may have decreased serum protein concentration. In fact, albumin reduced synthesis was observed in pigs fed deoxynivalenol contaminated diet and such reductions were more pronounced after 28 days of deoxynivalenol

challenge [47]. Likewise, the increase in aspartate aminotransferase observed in pigs fed CYB in comparison to PC is indicative of liver injury by leakage of the intracellular enzyme, since aspartate aminotransferase is the most sensible enzyme for liver injury in swine [48]. Deoxynivalenol also has a toxic effect on the kidney. The mycotoxin can impair kidney function by increasing serum creatinine and blood urea nitrogen, augment oxidative stress by increasing malondialdehydes and reducing superoxide dismutase activity, and eventually triggering renal cell apoptosis in mice [49]. There was a lower calcium concentration in serum of pigs fed CYB than pigs fed PC, suggesting that deoxynivalenol may have caused the hypocalcemia observed, due to its effects on the kidney. In addition, vitamin D deficiency related to liver malfunctioning induced by deoxynivalenol was related to lower calcium absorption leading to hypocalcemia [50]. Indeed, deoxynivalenol toxicity on kidney and liver health seems to have lasted until the end of the study on d 34 in pigs fed CYB, as seen for the lower calcium levels in comparison to pigs fed PC. On d 34, pigs fed CYC seemed to have improved liver and gut health, as seen by the lower creatine phosphokinase and higher glucose than pigs fed PC. As discussed previously, yeast culture metabolites provided by CYC may have improved gut integrity [31], then improving nutrient digestibility [32]. The improvement in liver health could be the eventual improvement by reduced mycotoxin absorption and its systemic toxicity observed at the liver. On the other hand, pigs fed CYE showed higher blood urea nitrogen to creatinine ratio than pigs fed PC, indicating impaired liver and kidney health. Such a result reinforces the increase observed in fecal score in pigs fed CYE in comparison to pigs fed PC, where there is an indication that yeast fermentation products may pose a bigger role in deoxynivalenol detoxification at the gastrointestinal level than the yeast cell wall alone.

The mid-jejunum (3.5 m distal from duodenum [51]) was targeted to evaluate the impacts of deoxynivalenol, because mid-jejunum is the major site for the absorption of deoxynivalenol [52] and the most important for nutrient absorption, and, thus, growth of pigs [53]. The tendency of an increase in malondialdehydes concentration, along with the decrease in total glutathione in pigs fed PC versus NC, is indicative of the fact that jejunal mucosa was under oxidative stress, due to a higher proportion of damaged cell components (malondialdehydes) and lower levels of antioxidant molecules (total glutathione). Similar results were found in another study, where deoxynivalenol intake culminated in increased malondialdehydes and decreased superoxide dismutase activity, another antioxidant enzyme [49]. Moreover, the glutathione-S-transferase plays a role in phase II detoxification of deoxynivalenol and, thus, depletion in total glutathione could be explained by its employment on for excretion of deoxynivalenol [12]. Nevertheless, such effects could not affect other oxidative stress markers, as protein carbonyls and immunological responses on tumor necrosis factor-alpha, interleukin 8, immunoglobulin A, and immunoglobulin G when comparing pigs fed NC and PC. Possibly, the chronic exposure to deoxynivalenol allowed the animals to overcome the mycotoxin toxicity and only mild effects could be observed after 34 days of exposure. Pigs fed the mycotoxin detoxifiers CYC and CYB presented lower interleukin 8 and lower immunoglobulin A, respectively, in comparison to pigs fed PC. In accordance with what was previously hypothesized, supplementation with *S. cerevisiae* either as yeast culture (CYC) or as inactivated yeast and fermentation extract (CYB) may have had an improved immune response. The yeast cell wall (CYE) did not show any immune-modulatory outcome in comparison to PC, suggesting that the whole cell was critical to trigger immunological effects in newly-weaned pigs challenged with deoxynivalenol in the current study. One yeast-derived component that may be absent in CYE that has previously

shown immune-modulatory activity and improving liver health and gut function is nucleotides. Yeast-derived nucleotides have a role in gut development in pigs, increasing enzyme activity in the stomach (pepsin) and proximal small intestine (alkaline phosphatase) [54]. In the same study, nucleotide supplementation increased plasmatic immunoglobulin A and influenced lymphocyte proliferation in lymphoid tissue. Besides, nucleotide beneficial effects can be observed during rapid growth and, especially, in the case of liver injury [55], and may enhance hepatic glucuronidation [56] for further deoxynivalenol detoxification. Thus, such results indicate that yeast fermentation products may pose a bigger role as deoxynivalenol detoxifiers, by reducing immune response in pigs challenged with the mycotoxin. Additionally, pigs fed CYB showed lower malondialdehydes and higher total glutathione, explaining the reduced cell damage by increasing antioxidant molecules that can repair deoxynivalenol injuries at the cellular level or be used for deoxynivalenol clearance from the organism. The gut may host bacteria in charge of deoxynivalenol detoxification by de-epoxidation [57]. However, pig microbiome has a low ability to detoxify deoxynivalenol in comparison to other species [58] and most of it is restrained to the lower digestive tract, where the majority of deoxynivalenol intake was already absorbed [52,59]. The complete pathway of deoxynivalenol detoxification in the pig also involves hepatic metabolism [15]. The cytochrome P-450 takes the first detoxification phase in the liver for further processing by glutathione-S-transferase, glutathione peroxidase, catalase, or superoxide dismutase [60,61]. During phase II, the liver plays a controversial role in deoxynivalenol detoxification after oral intake. The major excretion route of deoxynivalenol is through urine (about 70%) in its intact form (95%) [62], with more significant detoxification occurring in the intestine [59,63], whereas other studies support hepatic glucuronidation, but with elevated individual variation [64,65]. Therefore, our results show that the maintenance of gut integrity is

critical in pigs challenged with deoxynivalenol, rather than the additive's binding ability. The presence of plant extracts and antioxidants in CYE and CYB may have accounted for the reduction in oxidative stress in the gut. Plant compositions (milk thistle, rosemary, licorice, and boldo) in CYB may reduce inflammation by decreasing tumor necrosis factor-alpha and, thus, increasing cell viability *in vitro* [66]. Particularly, rosemary or its extract have the proven ability to reduce oxidative stress, and inflammatory and immune activation, besides improving liver function under mycotoxin challenge [67,68]. Additionally, α- and β-D-glucans, as components of the yeast cell wall, may modulate immune function and selectively interact with the gut microbiome [69]. Such yeast cell wall effects may be potentiated by their properties in reducing oxidative stress [70,71], improving overall pig health.

The mild differences observed among pigs fed NC and pigs fed PC regarding oxidative stress markers and immunological responses are consistent with the results observed in the histomorphology of mid-jejunal sections and nutrient digestibility. Deoxynivalenol did not influence morphology or Ki-67 measurements performed in histology sections from the mid-jejunum of pigs among experimental treatments. Eventually, the maintenance in intestinal structure could conserve the apparent ileal digestibility for dry matter, gross energy, nitrogen, or ether extract in pigs among experimental treatments.

In summary, feeding deoxynivalenol at 3.2 mg/kg of feed compromised ADG (reduced 14%), ADFI (tended to reduce by 12.3%), and G:F (tended to reduce by 10.7%) of newly-weaned pigs. Besides, pigs fed deoxynivalenol diets showed increased oxidative stress in mid-jejunum by increased malondialdehydes and reduced total glutathione. Yeast-based mycotoxin detoxifiers maintained growth performance and liver health, and improved intestinal health by reducing malondialdehydes (for clay/yeast/antioxidants), enhancing total glutathione (for

clay/yeast/antioxidant/botanicals), and reducing immune activation (for clay/yeast/antioxidant/botanicals) in the mid-jejunum of pigs fed diets with deoxynivalenol at 3.2 mg/kg of feed.

3.5. Conclusions

In conclusion, feeding naturally contaminated diets with deoxynivalenol (1.2 vs. 3.2 mg/kg) reduced growth performance and increased gut oxidative stress in nursery pigs. Yeast-based mycotoxin detoxifiers improved gut health, but could not fully overcome toxic effects of deoxynivalenol diets regarding growth and liver health in nursery pigs, where the yeast-based mycotoxin detoxifier with functional components as mineral and organic adsorbents, antioxidants, immune modulators, and health and digestibility promoters, showed improvements on immune activation, and reduction in oxidative stress.

3.6. Materials and Methods

A protocol of this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC, USA). The experiment was conducted at the North Carolina State University Metabolism Educational Unit (Raleigh, NC, USA).

On day zero, 30 barrows and 30 gilts weaned at 27 days of age (8.20 ± 0.10 kg) were allotted to 5 dietary treatments (n = 12), based on a completely randomized block design according to sex and body weight (heavy, medium, and light). Pigs were assigned to individual pens with metal screen floors, equipped with a nipple drinker and a feeder. Each pig received one

of the following 5 diets for 34 days: (1) a diet with corn DDGS containing 1.2 mg/kg of deoxynivalenol (NC), (2) a diet with corn DDGS contaminated with deoxynivalenol to supplement 2 mg/kg deoxynivalenol (PC), (3) PC + clay/yeast culture based product, at 0.2% (CYC); (4) PC + clay/yeast cell wall/plant extracts/antioxidants based product, at 0.2% (CYE); and (5) PC + clay/inactivated yeast/botanicals/antioxidants based product, at 0.2% (CYB). Experimental diets were formulated to meet or exceed the nutrient requirements suggested by NRC (2012), following a 3-phase feeding program (Table 6). All experimental diets were sampled (from 9 different locations, totaling 2 kg per diet) and 200 g of each were sent to North Carolina Department of Agriculture (Raleigh, NC, USA) for proximate analysis, and another 200 g of each was sent to North Dakota State University Veterinary Diagnostic Laboratory (Fargo, ND, USA) for determination on mycotoxin concentration (Table 7). Mycotoxins were extracted from feed samples by acetonitrile:water (84:16, v/v), filtered, and then screened by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) for mycotoxin detection. The NC diet had deoxynivalenol because corn DDGS used in the NC diet also included deoxynivalenol. However, the concentration of deoxynivalenol in PC still had 2 mg/kg more deoxynivalenol than NC, as planned.

Table 7. Concentrations of detectable mycotoxins in conventional dried distillers grains with solubles (DDGS) or high in deoxynivalenol (DON) contamination DDGS used for diet formulation to newly-weaned pigs for 34 d, based on a 3-phase feeding program.

Mycotoxin, µg/kg	Corn DDGS	
	Conventional	High in DON
Aflatoxin B1	5.5	7.4
Aflatoxin B2	ND	ND
Aflatoxin G1	0.1	0.1
Aflatoxin G2	4.5	3.3
Ochratoxin A	2.0	2.1
Ochratoxin B	ND	1.7
Citrinin	57.0	88.0
Deoxynivalenol	2064	5643
3-acetyl-deoxynivalenol	21.7	97.0
15-acetyl-deoxynivalenol	550	2,009
Deoxynivalenol-3-glucoside	38.7	61.5
Nivalenol	5540	ND
Fusarenon-X	158	142
Beauvericin	1.0	0.4
Moniliformin	92.1	59.6
Fusaric acid	431	456
T2 toxin	6.0	1.7
HT2 toxin	100	254
Diacetoxyscirpenol	4.1	6.9
Neosolaniol	3.2	1.5
Fumonisin B1	479	199
Fumonisin B2	27.3	26.9
Fumonisin B3	5.0	9.7
Zearalenone	213	2,417
Patulin	17.5	9.9
Alternariol	21.2	47.4
Citreoviridin	2.1	2.9
Cyclopiazonic acid	2.7	2.5
Ergocornin	2.5	1.9
Ergocristine	145	100
Ergocryptine	26.1	115
Ergometrine	0.0	0.0
Ergosine	38.5	70.2
Ergotamine	217	242
Gliotoxin	438	2468
Lysergol	0.1	ND
Methylergonovine	0.5	0.7
Mycophenolic acid	0.3	0.7
Penicillic acid	7.0	5.0
Roquefortine C	0.2	0.3
Sterigmatocystin	1.0	0.9
Verruculogen	11.3	53.7
Wortmannin	17.7	8.7

Mycotoxin concentrations were measured at 37+ Lab (Alltech Inc., Nicholasville, KY, USA). ND, not detected.

Table 8. Feed ingredients, calculated, and analyzed composition of experimental diets with (PC) or without mycotoxins (NC), in a three-phase feeding program fed to newly-weaned pigs for 34 d, based on a 3-phase feeding program.

Item	Phase 1 (d 0 to 7)		Phase 2 (d 7 to 21)		Phase 3 (d 21 to 34)	
	NC	PC	NC	PC	NC	PC
Ingredient, %						
Ground corn	14.67	14.67	31.07	31.07	43.42	43.42
Corn DDGS	22.00	-	22.00	-	22.00	-
Corn DDGS with DON[1]	-	22.00	-	22.00	-	22.00
Soybean meal	16.00	16.00	19.00	19.00	30.00	30.00
Whey permeate	20.00	20.00	10.00	10.00	-	-
Cookie meal	10.00	10.00	5.00	5.00	-	-
Poultry meal	6.00	6.00	4.00	4.00	-	-
Blood plasma	5.00	5.00	3.00	3.00	-	-
Fish meal	2.00	2.00	-	-	-	-
Poultry fat	2.00	2.00	3.00	3.00	2.00	2.00
Limestone	0.90	0.90	1.05	1.05	1.15	1.15
Dicalcium phosphate	-	-	0.50	0.50	0.70	0.70
Salt	0.22	0.22	0.22	0.22	0.22	0.22
L-lysine HCl	0.53	0.53	0.51	0.51	0.30	0.30
DL-methionine	0.15	0.15	0.12	0.12	0.02	0.02
L-threonine	0.10	0.10	0.10	0.10	0.01	0.01
Mineral mix	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin mix	0.03	0.03	0.03	0.03	0.03	0.03
Titanium dioxide	-	-	-	-	0.05	0.05
Calculated composition						
DM, %	91.10	91.10	90.48	90.48	89.57	89.57
ME, kcal/kg	3471	3471	3480	3480	3391	3391
SID Lys, %	1.504	1.504	1.349	1.349	1.228	1.228
SID Thr, %	0.876	0.876	0.800	0.800	0.732	0.732
SID Trp, %	0.246	0.246	0.221	0.221	0.232	0.232
SID Met+Cys, %	0.817	0.817	0.744	0.744	0.680	0.680
Ca, %	0.849	0.849	0.799	0.799	0.711	0.711
STTD P, %	0.469	0.469	0.407	0.407	0.334	0.334
Analyzed mycotoxin[2], mg/kg						
Zearalenone	0.179	0.355	0.156	0.244	ND	0.266
Deoxynivalenol	1.262	3.015	1.265	3.027	1.131	3.561
Fumonisin B1	ND	ND	ND	ND	0.238	0.203

NC, negative control diet formulated with DDGS contaminated with 2.6 mg/kg of deoxynivalenol; PC, positive control diet formulated with DDGS contaminated with 7.6 mg/kg of deoxynivalenol; ND, not detected. [1] DON corn DDGS, deoxynivalenol contaminated corn DDGS (mycotoxin concentration: 7.6 mg/kg of feed of deoxynivalenol, 0.2 mg/kg of feed of fumonisin B1, and 0.5 mg/kg of feed of fusaric acid). [2] Mycotoxin concentrations were measured by LC-MS/MS Screen at the North Dakota State University Veterinary Diagnostic Laboratory (Fargo, ND, USA). Ingredients that were not included in a given formula have a dash (-) on its respective column.

The body weights of pigs and feed disappearances by pigs were recorded weekly to calculate average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F). The pig fecal score was recorded on days 5, 7, and 14 [72]. On days 14 and 34, blood samples of were collected using 0.8 × 32 mm needles (Eclipse, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) from the jugular vein, in 10 mL collection tubes for blood serum (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). After clot development, collection tubes were centrifuged at 1509 × *g* at 4 °C for 15 min (5811F, Eppendorf, Hamburg, HH, Germany). The supernatant, serum, was transferred to 1.5 mL tubes (Fisherbrand, Fisher Scientific, Hampton, NH, USA) and samples were stored at −80 °C in a freezer (812660-760, Thermo Fisher Scientific, Waltham, MA, USA). Serum samples were submitted to assess proteins, metabolites, and electrolytes at a commercial laboratory (Antech Diagnostic Laboratory, Cary, NC, USA).

At the end of the study, d 34, pigs were desensitized by penetrating captive bolt and euthanized by exsanguination of the vena cava. Intestinal samples were collected 3.5 m after the duodenum, delimited by the end of the anatomical association between duodenum and pancreas, and considered as mid-jejunum [51]. Samples of gut mucosa were scrapped from 15 cm sections from mid-jejunum and stored at −80 °C until laboratory analysis. Antioxidant status, immune response, and intestinal barrier function were evaluated in gut mucosa by quantifying protein carbonyls (STA-310, Cell Biolabs, Inc., San Diego, CA, USA), malondialdehydes (STA-330, Cell Biolabs, Inc., San Diego, CA, USA), total glutathione (STA-312, Cell Biolabs, Inc., San Diego, CA, USA), tumor necrosis factor-alpha (PTA00, R&D Systems, Inc., Minneapolis, MN, USA), interleukin 8 (P8000, R&D Systems, Inc., Minneapolis, MN, USA), immunoglobulin G (E100-104, Bethyl Laboratories, Inc., Montgomery, TX, USA), and immunoglobulin A (E100-

102, Bethyl Laboratories, Inc., Montgomery, TX, USA). The protocols provided by the kit manufactures were followed for determining the relative concentrations to protein content (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) determined from the same samples.

Ileal digesta collected on d 34 was stored at −80 °C until processing for lab analysis: freeze-drying and grinding. Dried and ground ileal digesta samples were analyzed for the apparent ileal digestibility of dry matter [73], gross energy (6200 Calorimeter, Parr Instrument Company, Moline, IL, USA), nitrogen (AOAC method 990.03, [74]), and ether extract (AOAC method 920.39, [74]).

A fragment of gut tissue (5 cm) from mid-jejunum was fixed in 10% buffered formalin for histological evaluation. Transversal sections from mid-jejunum were sent to the North Carolina State University Histopathology Laboratory (College of Veterinary Medicine, Raleigh, NC, USA) for inclusion in paraffin and immunohistochemistry staining for Ki-67 antigen [75]. The evaluation of mid-jejunal morphology in histological sections was assessed by one evaluator by measuring villus height and width, crypt depth, villus height:crypt depth ratio, and for estimating the percentage of proliferating cells in the crypt after Ki-67 staining [76] in ten pictures of each pig (Figure 4). The percentage of proliferating cells is calculated by dividing the nuclear area of cells positive to the Ki-67 antigen by the nuclear area of all cells in the crypt.

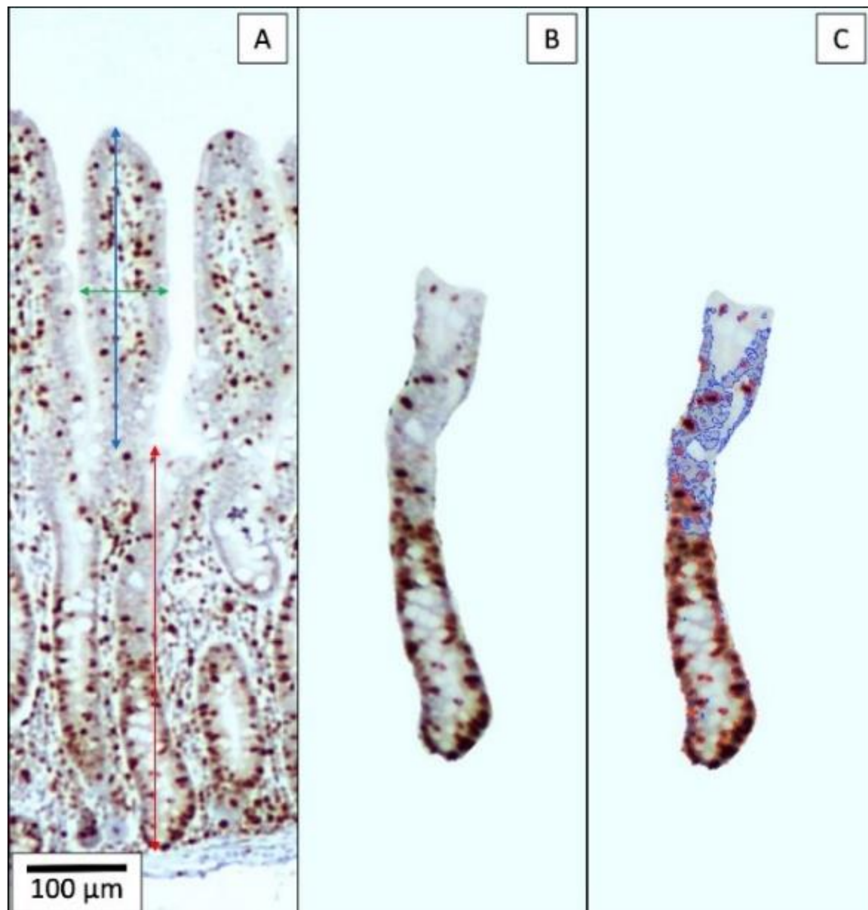

Figure 4. Ten microscopical images (40×) of a well-oriented villus and its associated crypt (**A**) were obtained for measuring villus height (double arrow line in blue), villus width (double arrow line in green), and crypt depth (double arrow line in red). This was followed by cropping the crypt (**B**) and assessing the nuclei of cells positive to Ki-67 staining (delimited in red, **C**) in proportion to total cell number (sum of nuclei delimited in blue and red). (**C**) was obtained using ImageJS tool [76].

The statistical analysis was performed by using the MIXED procedure of SAS 9.3 software (Statistical Analysis System, Cary, NC, USA, 2011). The experimental unit was considered as a pen (one pig). Blocks (sex and initial body weight) were considered as random effects. The effect of sex was checked by assessing the interaction among treatments and sex using the MIXED procedure, but no effect was observed. Analyses of pre-planned contrasts between NC and PC as well as PC and mycotoxin detoxifiers (CYC, CYE, and CYB) were performed using the CONTRAST statement for comparisons with F test. The LSMEANS

statement was used for the separation of means. Results were considered statistically different for $p < 0.05$ and $0.05 \leq p < 0.10$ were considered tendency. The design was based on the power test using previous studies conducted under similar measures and objectives (n = 12 was the minimum, considering a large individual variation).

3.7. References

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CHAPTER 4. INVESTIGATION OF THE EFFICACY OF A MYCOTOXIN-DETOXIFYING ADDITIVE ON HEALTH AND GROWTH OF NEWLY-WEANED PIGS UNDER DEOXYNIVALENOL CHALLENGES

Holanda, D. M., and S. W. Kim. 2020. Investigation of the efficacy of a mycotoxin-detoxifying additive on health and growth of newly-weaned pigs under deoxynivalenol challenges. Asian-Australasian Journal of Animal Science. (Accepted).

4.1. Abstract

Objective: This study evaluated the effects of feeding diets naturally contaminated with deoxynivalenol (supplemental 2 mg/kg) on health, growth, and the effects of a mycotoxin-detoxifying additive in newly-weaned pigs.

Methods: Thirty-six pigs (27 day-old) were housed individually and assigned to 3 treatments for 5 weeks: CON (diet containing minimal deoxynivalenol), MT (diet with supplemental 1.9 mg/kg of deoxynivalenol), and MT+D (MT + mycotoxin-detoxifying additive, 0.2%, MegaFix®, ICC, São Paulo, Brazil). The mycotoxin-detoxifying additive included bentonite, algae, enzymes, and yeast. Blood was taken at weeks 2 and 5. Jejunal tissues were taken at week 5. Data were analyzed using the MIXED procedure of SAS.

Results: Pigs fed MT+D tended to have decreased ($p = 0.056$) averaged daily feed intake during week 1 than MT. At week 2, serum aspartate aminotransferase/alanine aminotransferase in MT tended to be lower ($p = 0.059$) than CON, whereas it was increased ($p < 0.05$) for MT+D than MT, indicating hepatic damages in MT and recovery in MT+D. Pigs fed MT had lower ($p < 0.05$) blood urea nitrogen/creatinine than CON, supporting hepatic damage. At week 5, pigs fed MT tended to have reduced ($p = 0.079$) glucose than CON, whereas it was increased ($p < 0.05$) for MT+D than MT, indicating impaired intestinal glucose absorption in MT, which was improved in MT+D. Pigs fed CON tended to have increased ($p = 0.057$) total glutathione in jejunum than MT, indicating oxidative stress in MT. Pigs fed MT+D had a reduced ($p < 0.05$) proportion of Ki-67-positive cells in jejunum than MT, indicating lower enterocyte proliferation in MT+D.

Conclusion: Feeding supplemental 1.9 mg/kg of deoxynivalenol reduced growth and debilitated hepatic health of pigs, as seen in leakage of hepatic enzymes, impaired nitrogen metabolism, and

increase in oxidative stress. The mycotoxin-detoxifying agent enhanced hepatic health and glucose levels, and attenuated gut damage in pigs fed deoxynivalenol contaminated diets.

Keywords: adsorbent; deoxynivalenol; enzyme; gut health; pig; probiotic

4.2. Introduction

Mycotoxins are secondary metabolic products of fungi with toxigenic effects in other living species. Worldwide, about 88% of animal feed and feedstuffs are contaminated with at least one mycotoxin [1]. *Fusarium* toxins are the most prevalent, where deoxynivalenol ranks at first with 64% of occurrence, followed by fumonisins and zearalenone [1].

In pigs chronically fed deoxynivalenol contaminated diets, it is observed increased expression of interleukin 8 and glutathione peroxidase [2]. Deoxynivalenol also has shown impairment in the translation of mRNA that may ultimately affect cell proliferation, immune response, development, death [3–5], oxidative stress, and reduction in feed intake and growth of pigs [6,7]. Pigs challenged with naturally contaminated diets with deoxynivalenol showed impaired gut health by reducing enterocyte proliferation and intestinal surface area, resulting in compromised weight gain [8].

The chemical properties of deoxynivalenol make it difficult to be adsorbed or neutralized by mycotoxin-detoxifying additives. Thus, microorganisms, inorganic compounds, aluminosilicates, and yeast-based products are tested aiming to reduce deoxynivalenol toxicity [9–12] as in the mycotoxin-detoxifying additive tested in the current study. The aluminosilicates, such as the bentonite, have proven ability to adsorb mycotoxins based on the electrical charge of molecules [13,14]. Similarly, autolyzed yeast has demonstrated higher specificity for deoxynivalenol adsorption in comparison to mineral adsorbents [13,15]. At the same time, the extensive use of *Saccharomyces cerevisiae* with neutral or beneficial outcomes, and its availability turns its utilization feasible and positive to the livestock feed industry [16]. Of interest, *S. cerevisiae* was able to reduce inflammation and oxidative stress and to increase cell survivability in deoxynivalenol challenged pigs [17]. In association with those, the tested

mycotoxin-detoxifying additive has an enzyme complex composed of esterase, epoxide-reductase, and peptidase can detoxify deoxynivalenol [18], and other *Fusarium* toxins [19]. The algae powder derived from *Lithothamnium calcareum* has shown *in vitro* adsorbability towards zearalenone [20] and both *in vitro* and *in vivo* (broilers) toward aflatoxin B1 [21] but no studies have investigated its efficacy towards deoxynivalenol to date. Thus, the use of mycotoxin detoxifiers with multiple components, targeting a synergistic effect, is preferred to boost detoxification effects [12,22].

After analyzing publications on deoxynivalenol challenges in pigs, it was observed that each supplemental mg/kg of the mycotoxin in the feed would result in an 8% decrease in animal growth [23]. Thus, it is hypothesized that deoxynivalenol supplemented at 2 mg/kg would impair the health and growth of newly-weaned pigs and that using a mycotoxin-detoxifying additive including adsorbents (aluminosilicates and yeast cell wall - β-glucans), antioxidants, and immune modulators (algae and yeast as probiotic), and components to inactivate mycotoxins (enzymes), would attenuate the negative impacts of deoxynivalenol to newly-weaned pigs. The objective of this study was to evaluate the effects feeding diets naturally contaminated with deoxynivalenol (supplemental 2 mg/kg) on health and growth and the efficacy of a mycotoxin-detoxifying additive in mitigating such effects in newly-weaned pigs.

4.3. Materials and Methods

The Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC, USA) reviewed and approved a protocol for conducting this study at the North Carolina State University Metabolism Educational Unit (Raleigh, NC, USA).

4.3.1. Animals and diets

Upon weaning at 27 d of age (d 0), 36 barrows and gilts (8.21 ± 0.54 kg) were individually assigned to 3 dietary treatments based on a randomized complete block design. Blocks were sex (male and female) and initial body weight (light, medium, and heavy). Pigs were individually housed in pens with metal screen floors with individual nipple drinkers and feeders. Each pig was fed one of the 3 dietary treatments for 34 days: (1) a control diet with minimal deoxynivalenol contamination (CON), (2) CON with a supplemental 1.9 mg/kg of deoxynivalenol (MT), and (3) MT + mycotoxin-detoxifying additive (MegaFix®, ICC, São Paulo, Brazil) at 0.2% (MT+D). Based on the safety of yeast additives in swine diets (over 10 years) [16], and the effect of similar mycotoxin detoxifiers (adsorbent and yeast-based products) have been previously tested [8,24], this study was not to test the effects of mycotoxin-detoxifying additive in a control diet but only in a diet with mycotoxin [12]. The CON diet was formulated with “clean corn DDGS”, minimal mycotoxin contamination, whereas MT was formulated with “deoxynivalenol contaminated corn DDGS” (Table 1). The nutrient requirements suggested by NRC (National Research Council, 2012) were used to formulate experimental diets (Table 2). Diets followed a 3-phase feeding program: phase 1, during 1 week; phase 2, from week 2 until week 3; and phase 3, from week 4 until week 5. Phase 3 was shortened in 1 day for better arranging sampling schedule, so week 5 lasted for 6 days.

Table 1. Concentrations of selected mycotoxins in clean corn DDGS or deoxynivalenol contaminated corn DDGS used for experimental diets.

Mycotoxin, mg/kg	Clean corn DDGS	Contaminated corn DDGS
Deoxynivalenol	2.620	7.643
Fumonisin B1	0.480	0.200
Zearalenone	0.213	2.417

Mycotoxin concentrations were measured at 37+ Lab (Alltech Inc., Nicholasville, KY).

Table 2. Feed ingredients and calculated composition of experimental diets in a 3-phase feeding program fed to newly-weaned pigs for 5 weeks[1)]

Item	Phase1 (week 1)	Phase2 (weeks 2 and 3)	Phase3 (weeks 4 and 5)[2)]
Ingredient, %			
Corn	14.72	31.12	43.22
Corn DDGS[3)]	22.00	22.00	22.00
Soybean meal	16.00	19.00	30.00
Whey permeate	20.00	10.00	0.00
Bakery meal	10.00	5.00	0.00
Poultry meal	6.00	4.00	0.00
Blood plasma	5.00	3.00	0.00
Fish meal	2.00	0.00	0.00
Poultry fat	2.00	3.00	2.00
Limestone	0.90	1.05	1.14
Dicalcium phosphate	0.00	0.50	0.70
L-lysine HCl	0.53	0.51	0.30
DL-methionine	0.15	0.12	0.02
L-threonine	0.10	0.10	0.01
Salt	0.22	0.22	0.22
Mineral premix	0.15	0.15	0.15
Vitamin premix	0.03	0.03	0.03
Titanium dioxide	0.00	0.00	0.05
Additive	0.20[4)]	0.20[4)]	0.20[4)]
Calculated composition			
Dry matter, %	91.10	90.48	89.57
ME[5)], kcal/kg	3471	3480	3391
SID[6)] lysine, %	1.504	1.349	1.228
SID threonine, %	0.876	0.800	0.732
SID tryptophan, %	0.246	0.221	0.232
SID methionine+cysteine, %	0.817	0.744	0.680
Ca, %	0.849	0.799	0.711
STTD[7)] P, %	0.469	0.407	0.334
Analyzed composition[8)]			
Dry matter, %	94.04	93.43	93.01
Ca, %	0.865	0.775	0.704
STTD P, %	0.547	0.491	0.346

[1)] MegaFix® (ICC, São Paulo, Brazil) was added to MT at 0.2% in all phases.
[2)] Week 5 lasted 6 days to facilitate the sampling schedule.
[3)] Either "clean" corn DDGS or deoxynivalenol contaminated corn DDGS (mycotoxin concentration: 7.643 mg/kg of feed of deoxynivalenol, 0.200 mg/kg of feed of fumonisin B1, and 2.417 mg/kg of feed of zearalenone) was used to formulate CON or MT diets, respectively.
[4)] Either corn (CON and MT) or the mycotoxin-detoxifying additive (MT+D) was added to diets.
[5)] ME, metabolizable energy.
[6)] SID, standardized ileal digestible.
[7)] STTD, standardized total tract digestible.
[8)] The analyzed composition values are given on an as-fed basis.

Growth performance was recorded weekly by measuring body weight of pigs and feed consumption by pigs to obtain averaged daily gain, averaged daily feed intake, and to calculate gain to feed ratio. Fecal score of pigs was recorded on d 5, and at the end of week 1 and week 2 by ranking feces on a scale from 1 to 5 by a single evaluator. Feces considered in level 1 on the proposed scale are considered "normal" and have increasing water content until reaching level 5. In level 2, feces have increased water content but the characteristic shape of feces is maintained. In level 3, feces start to loose their characteristic shape, frequently with liquid and solid components mixed. In level 4, feces become viscous and pasty, with no vertical structure. In level 5, feces are liquid and do not have any consistency. At the end of week 2 and week 5, the external jugular vein was punctured using needles (0.8 x 32 mm; Eclipse, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) to obtain 10 mL of blood samples into serum blood collection tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Blood samples were allowed to clot for 4 hours at room temperature, blood samples were centrifuged at 1,500x g at 4°C for 15 minutes (5811F, Eppendorf, Hamburg, HH, Germany) to obtain blood serum. Blood serum samples were stored at -80°C freezer (812660-760, Thermo Fisher Scientific, Waltham, MA, USA) in 1.5 mL microtubes (Fisherbrand, Fisher Scientific, Hampton, NH, USA) until laboratory analyses.

By the end of week 5, a penetrating captive bolt was used to desensitize pigs and they were euthanized by immediate vena cava section for the collection of mid-jejunal mucosa and tissue. A mid-jejunal section of 15 cm was scrapped with a clean histological slide to obtain mid-jejunal mucosa [12]. Mucosa samples were stored 1.5 mL microtubes at -80°C until laboratory analyses. A second tissue section of 5 cm from mid-jejunum was fixed in 10% buffered formaldehyde and stored at room temperature.

4.3.2. *Assay procedures*

Diets from each experimental and within each phase were sampled from 9 different locations, a total of 2 kg per diet. Sub-samples of 200 g of each diet and phase were sent to the North Dakota State University Veterinary Diagnostic Laboratory (Fargo, ND, USA) for mycotoxin analysis, and 300 g of each diet were sent to the North Carolina Department of Agriculture (Raleigh, NC, USA) for proximate analysis. The CON had detectable deoxynivalenol levels because corn DDGS used in CON formulation had deoxynivalenol contamination. Nevertheless, MT had 1.9 mg/kg more deoxynivalenol than CON, close to the planned concentration of 2 mg/kg.

Serum samples were submitted for a biochemical profile at Antech Diagnostic Laboratory (Cary, NC, USA). Antioxidant status and immune markers were evaluated in mid-jejunal mucosa by quantifying protein carbonyls (STA-310, Cell Biolabs, Inc., San Diego, CA, USA), malondialdehydes (STA-330, Cell Biolabs, Inc., San Diego, CA, USA), total glutathione (STA-312, Cell Biolabs, Inc., San Diego, CA, USA), tumor necrosis factor-alpha (PTA00, R&D Systems, Inc., Minneapolis, MN, USA), and interleukin-8 (P8000, R&D Systems, Inc., Minneapolis, MN, USA). The manufacturer's manual for each kit was followed in the laboratory assays of protein carbonyls, malondialdehydes, and total protein according to procedures described by Zhao and Kim [26]. Measurements of total glutathione, tumor necrosis factor-α, and interleukin-8 followed the procedures as described by Holanda et al. [24].

Transversal sections of 0.5 cm were transferred to 70% ethanol after 14 days and sent to the North Carolina State University Histopathology Laboratory (College of Veterinary Medicine, Raleigh, NC, USA), where samples were included in paraffin, microtomed, and stained for Ki-67 antigen by immunohistochemistry before assembling of histological slides [8]. Histological

evaluation of gut morphology was performed by one single evaluator recording villus height and width, crypt depth, and for calculating villus height:crypt depth ratio, and the proportion of proliferating cells to the total number of cells in the crypt using the Image JS tool [27] in ten pictures for each experimental unit (pig) according to the measurements described by Holanda and Kim [12]. Measurements of villus height and width were used to calculate the average mid-jejunal surface area for each villus by using the following formula [28]:

$$Surface\ area = 2\pi \frac{villus\ width}{2}(villus\ height) + \pi \left(\frac{villus\ width}{2}\right)^2$$

4.3.3. Statistical analysis

The statistical analysis was performed using the Mixed procedure of SAS 9.3 software (Cary, USA). Each pig was considered as one experimental unit. Blocks (sex and initial body weight) were considered as random effects. Analyses of pre-planned contrasts between CON and MT as well as MT and MT+D were performed using the Contrast statement. Results were considered statistically different for $p < 0.05$ and considered a tendency for $0.05 \leq p < 0.10$. The design was based on the power test using previous studies conducted under similar conditions and objectives (n = 12 was minimum considering a large individual variation).

4.4. Results

The average mycotoxin contamination for the 3 dietary phases in CON samples was 1.2 mg/kg of deoxynivalenol, 0.21 mg/kg of fumonisin B1, and 0.15 mg/kg of zearalenone (Table 3). The average mycotoxin contamination for the 3 dietary phases in MT samples was 3.1 mg/kg of deoxynivalenol, 0.20 mg/kg of fumonisin B1, and 0.30 mg/kg of zearalenone.

Table 3. Concentrations of detected mycotoxins in experimental diets with (MT[1)]) or without mycotoxins (CON) fed to newly-weaned pigs for 5 weeks on a 3-phase feeding program[2)]

Mycotoxin, mg/kg of feed	Phase1 (week 1)			Phase2 (weeks 2 and 3)			Phase3 (weeks 4 and 5)[3)]		
	CON	MT	MT+D	CON	MT	MT+D	CON	MT	MT+D
Deoxynivalenol	1.262	3.015	2.640	1.265	3.027	3.346	1.131	3.561	3.214
Fumonisin B1	0.200	0.200	0.200	0.200	0.200	0.200	0.238	0.203	0.214
Zearalenone	0.179	0.355	0.358	0.156	0.244	0.317	0.100	0.266	0.249

Mycotoxin concentrations were measured by liquid chromatography tandem mass spectrometry at the North Dakota State University Veterinary Diagnostic Laboratory (Fargo, ND, USA).
CON, control diet formulated with corn DDGS with minimal mycotoxin contamination; MT, diet formulated with corn DDGS with supplemental 5 mg/kg of deoxynivalenol contamination.
[1)] MT diets have 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS.
[2)] MegaFix® (ICC, São Paulo, Brazil) was added to MT at 0.2% in all phases to create another treatment, MT+D.
[3)] Week 5 lasted 6 days to facilitate the sampling schedule.

There were no differences in pig body weight among pigs from experimental treatments (Table 4). Pigs fed MT+D tended to have increased ($p = 0.099$) averaged daily gain than pigs fed MT during week 4 (Figure 1). Pigs fed MT tended to present lower averaged daily gain during week 5 ($p = 0.084$) than pigs fed CON. Pigs fed MT+D tended to have lower ($p = 0.099$) average daily feed intake than pigs fed MT during week 1. There were no differences for gain to feed ratio or fecal score (Table 5) among pigs from experimental treatments.

Table 4. Body weight of weaned pigs consuming experimental diets with (MT[1)]) or without mycotoxins (CON) for 5 weeks.

Treatment	CON	MT	MT+D	SEM	*p* Value	
					CON vs MT	MT vs MT+D
Body weight, kg						
Initial	8.20	8.20	8.22	0.54	0.956	0.878
Week 1	8.55	8.52	8.33	0.52	0.923	0.424
Week 2	9.94	9.76	9.79	0.59	0.616	0.929
Week 3	12.98	12.60	12.53	0.69	0.543	0.909
Week 4	16.86	15.86	16.47	0.85	0.265	0.496
Week 5[2)]	21.30	19.77	20.63	1.03	0.163	0.429

There was no animal mortality during the experimental period.
CON, control diet formulated with corn DDGS with minimal mycotoxin contamination; MT, diet formulated with corn DDGS with supplemental 5 mg/kg of deoxynivalenol contamination; and MT+D, MT + MegaFix® (ICC, São Paulo, Brazil) at 0.2%.
[1)] MT and MT+D diets have 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS.
[2)] Week 5 lasted 6 days to facilitate the sampling schedule.

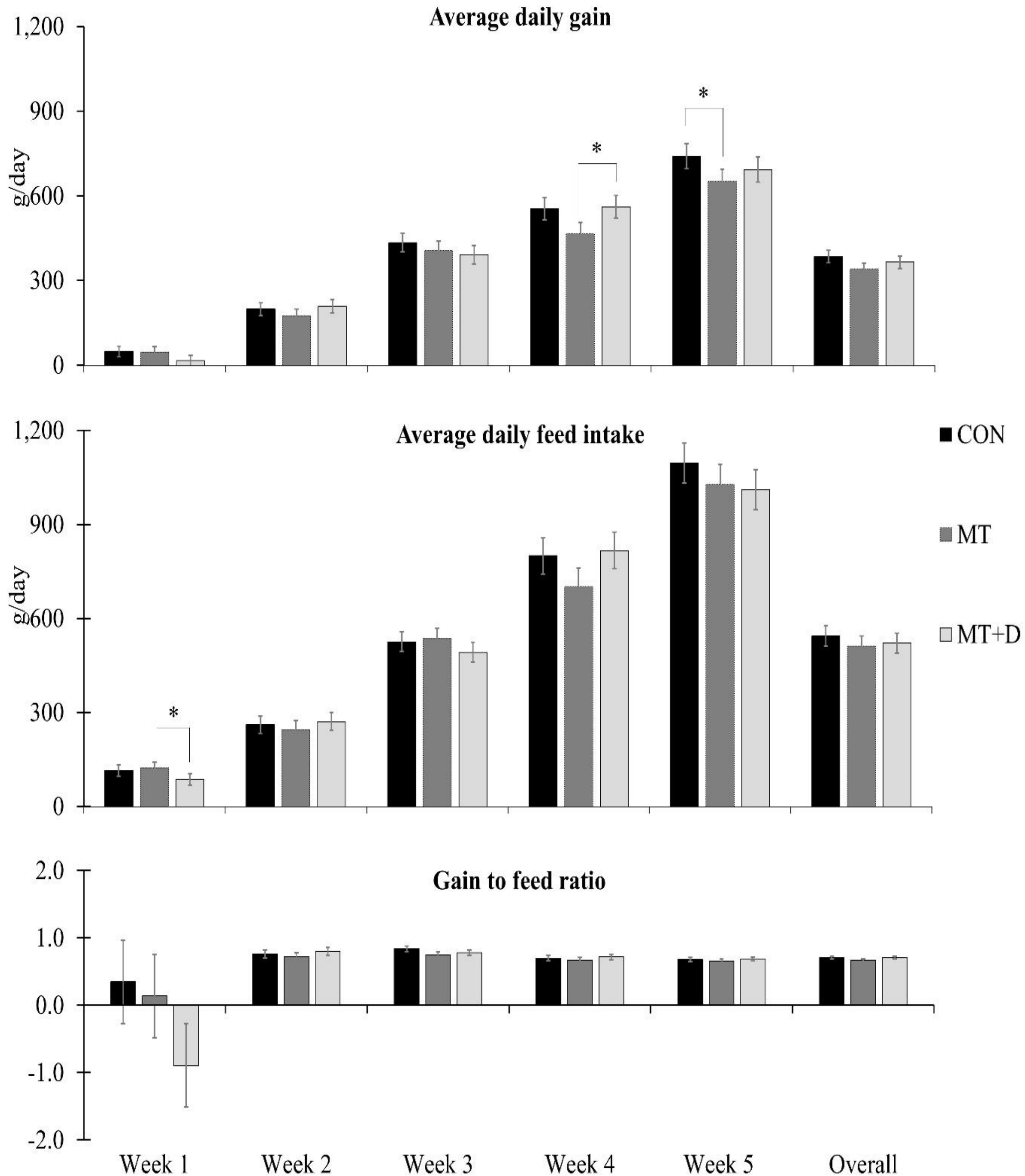


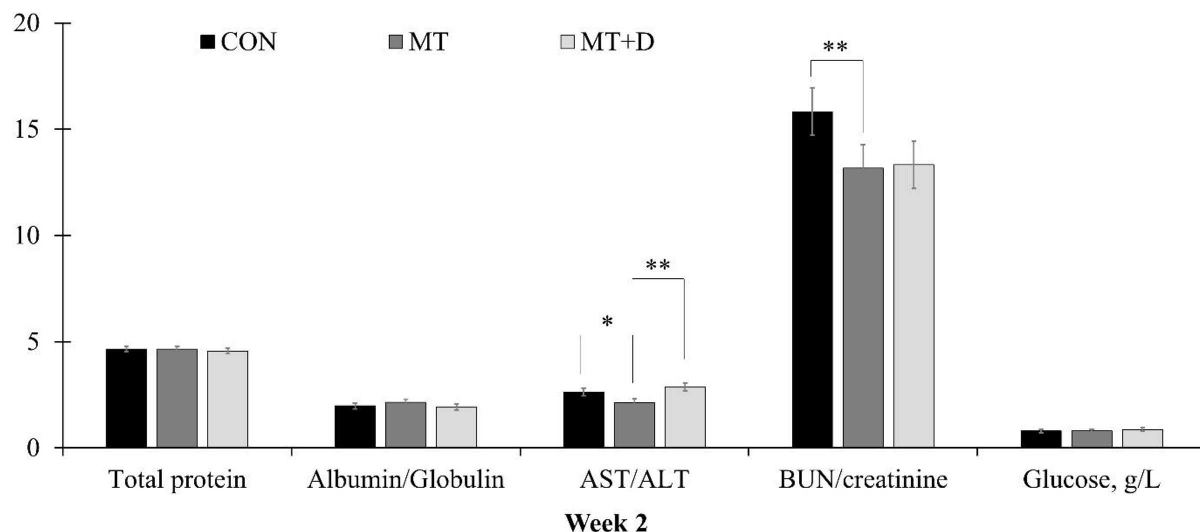
Figure 1. Average daily gain, average daily feed intake, and gain to feed ratio observed in weaned pigs consuming experimental diets with (MT) or without mycotoxins (CON) for 5 weeks. CON, control diet formulated with minimal deoxynivalenol contamination; MT, CON + 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS; MT+D, MT + mycotoxin-detoxifying additive (0.2% of MegaFix®; ICC, São Paulo, Brazil); and *, $0.05 \leq p < 0.10$. Week 5 lasted 6 days to facilitate the sampling schedule.

Table 5. Fecal score recorded in weaned pigs consuming experimental diets with (MT[1)]) or without mycotoxins (CON) at d 5, end of week 1, and end of week 2

Treatment	CON	MT	MT+D	SEM	*p* Value	
					CON vs MT	MT vs MT+D
Fecal score[2)]						
d 5	3.3	2.6	3.7	0.6	0.372	0.203
Week 1	2.5	2.1	3.0	0.5	0.480	0.157
Week 2	1.6	1.7	1.4	0.3	0.434	0.869

CON, control diet formulated with corn DDGS with minimal mycotoxin contamination; MT, diet formulated with corn DDGS with supplemental 5mg/kg of deoxynivalenol contamination; MT+D, MT + MegaFix® (ICC, São Paulo, Brazil) at 0.2%.
[1)] MT and MT+D diets have 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS.
[2)] Fecal score was subjectively measured based on a 1 to 5 scale.

By the end of week 2, pigs fed MT tended to have lower (p = 0.059) aspartate aminotransferase/alanine aminotransferase (AST/ALT) and lower (p < 0.05) blood urea nitrogen/creatinine than pigs fed CON (Figure 2). Pigs fed MT+D had increased (p < 0.05) AST/ALT than pigs fed MT. By the end of week 5, pigs fed MT tended to have reduced (p = 0.079) glucose in serum than pigs fed CON, whereas pigs fed MT+D had increased (p < 0.05) glucose levels than pigs fed MT. There were no differences in other variables assessed for biochemical profile among pigs from experimental treatments.



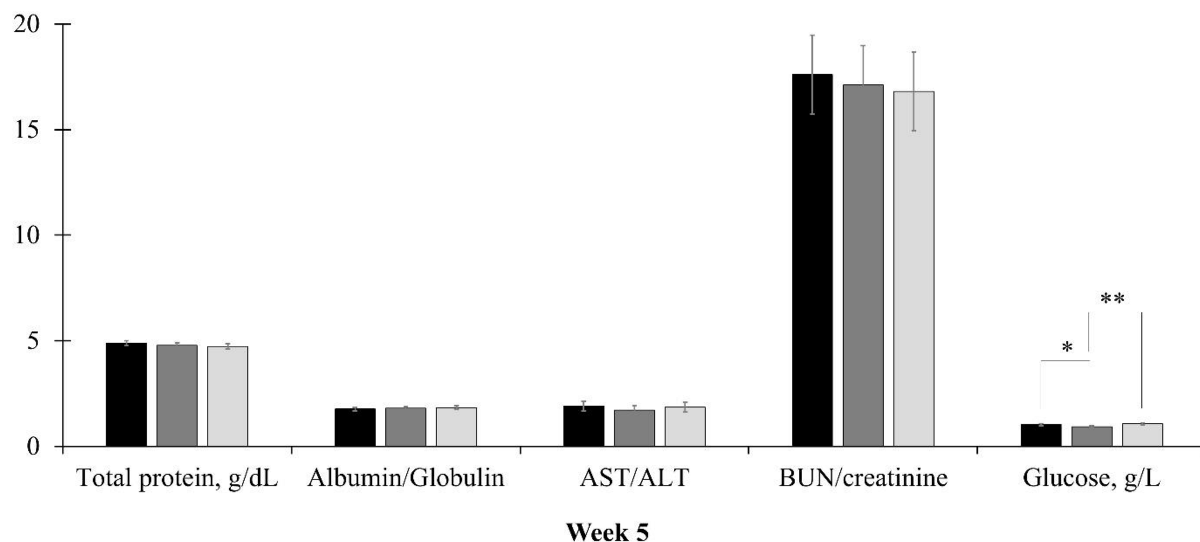


Figure 2. Serum variables observed in weaned pigs consuming experimental diets with (MT) or without mycotoxins (CON) by the end of week 2 and week 5. CON, control diet formulated with minimal deoxynivalenol contamination; MT, CON + 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS; MT+D, MT + mycotoxin-detoxifying additive (0.2% of MegaFix®; ICC, São Paulo, Brazil); AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; and *, $0.05 \leq p < 0.10$; and **, $p < 0.05$.

Pigs fed CON tended to have higher ($p = 0.057$) concentration of total glutathione in mid-jejunal mucosa than pigs fed MT (Figure 3). There were no differences in tumor necrosis factor-alpha, interleukin 8, protein carbonyl, or malondialdehydes in mid-jejunal mucosa of pigs among

experimental treatments. There were no differences in villus surface area, crypt depth, nor villus height to crypt depth ratio of pigs among experimental treatments (Table 6). Pigs fed MT+D had a lower ($p < 0.05$) proportion of proliferating cells measured by Ki-67 staining in histological slides from mid-jejunum than pigs fed MT.

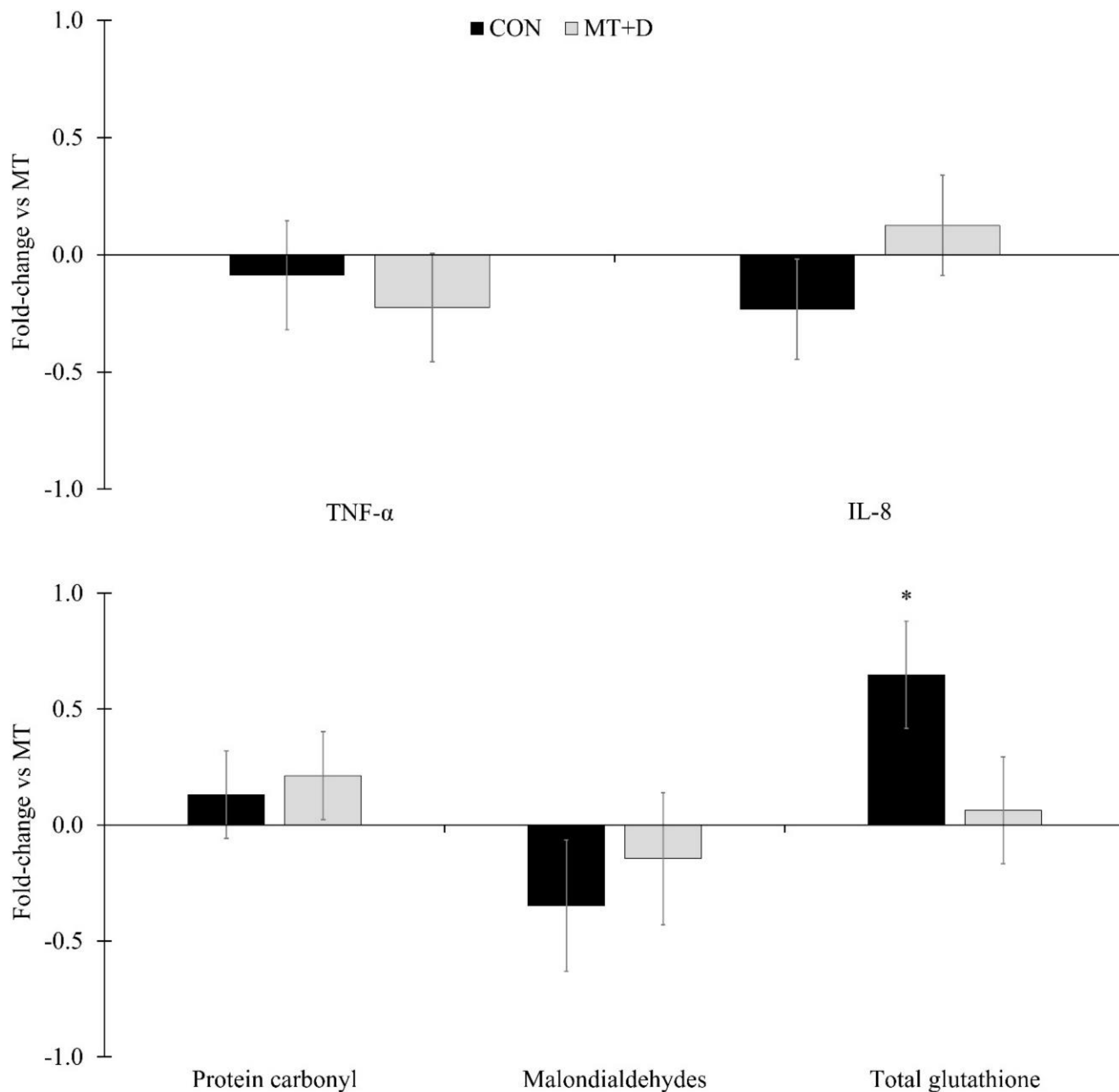


Figure 3. Inflammatory and oxidative stress markers in gut mucosa in weaned pigs consuming experimental diets with (MT) or without mycotoxins (CON) for 5 weeks expressed as fold-change relative to pigs fed MT. CON, control diet formulated with minimal deoxynivalenol contamination; MT, CON + 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS; MT+D, MT + mycotoxin-detoxifying additive (0.2% of MegaFix®; ICC, São Paulo, Brazil); TNF-α, tumor necrosis factor-alpha; IL-8, interleukin-8; and *, $0.05 \leq p < 0.10$.

Table 6. Histological measurement from mid-jejunal morphology and immunohistochemistry in weaned pigs consuming diets with (MT[1)]) or without mycotoxins (CON) and diet with mycotoxins and feed additives by the end of week 5

Treatment	CON	MT	MT+D	SEM	*p* Value	
					CON vs MT	MT vs MT+D
Villus surface area x10^3, μm^2	254.9	242.3	216.6	23.8	0.625	0.321
Crypt depth (CD), µm	256.5	249.5	229.9	12.3	0.622	0.174
Villus height/CD	1.64	1.79	1.62	0.24	0.425	0.381
Ki-67[2)], %	29.43	27.23	21.62	1.92	0.191	0.002

CON, control diet formulated with corn DDGS with minimal mycotoxin contamination; MT, diet formulated with corn DDGS with supplemental 5mg/kg of deoxynivalenol contamination; and MT+D, MT + MegaFix® (ICC, São Paulo, Brazil) at 0.2%.

[1)] MT and MT+D diets have 1.9 mg/kg of deoxynivalenol supplemented from mycotoxin contaminated corn DDGS.

[2)] Ki-67 counting is an estimate of the proliferative rate, calculated based on the proportion of cells positive to Ki-67 immunohistochemistry to the total cell number.

4.5. Discussion

As intended in the current study (supplemental 2 mg/kg), the MT diet had a supplemental 1.9 mg/kg of deoxynivalenol in comparison to CON. The intended concentration in MT aimed to surpass the guidelines proposed for this mycotoxin, for instance, 1 mg/kg in the United States [29] and 0.9 mg/kg in Europe [30] for growing pigs. Other mycotoxins frequently found as co-contaminants with deoxynivalenol were detected, fumonisin B1 and zearalenone [1]. Fumonisin B1 concentration did not exceed the guidance for growing pigs in the United States nor Europe [29,30] neither in CON nor MT. Zearalenone concentration in CON (0.15 mg/kg) and MT (0.30 mg/kg) exceeded the guidance levels in Europe of 0.1 mg/kg [30]. The European guidance levels include both young pigs (growing) and gilts (reproduction), but the latter group of animals was not the focus of the current study. Regarding growing animals, zearalenone effects include improved feed intake and growth [31] equivalent growth performance between challenged and unchallenged animals [32]. However, in naturally contaminated diets (along with other *Fusarium*

toxins) a detrimental effect of zearalenone can be observed on pig growth [33]. Because of the controversial effects of zearalenone on pig growth in scientific publications, the effects of this mycotoxin will not be considered for further discussion.

The dietary intake of deoxynivalenol is known to reduce feed intake and gain of pigs [34]. The reduced feed intake may be caused by deoxynivalenol anorexigenic and emetic effects. It was described that deoxynivalenol mediated increase in cholecystokinin and, more significantly, in peptide YY is claimed as the mechanisms to result in reduced feed intake [35]. However, the current study could not demonstrate a consistent impairment in pig growth performance. Besides the reduction in averaged daily gain during week 5 in pigs fed MT in comparison to pigs fed CON, there was no further deoxynivalenol effect observed on growth performance. Pigs show reduced growth performance when fed from 1 to 3 mg/kg of deoxynivalenol [34]. In the current study, the CON diet had 1.2 mg/kg whereas the MT diet had 3.1 mg/kg of deoxynivalenol. Therefore, possibly pigs had some degree of growth impairment when fed the CON diet and only a moderate further decrease in growth performance could be noticed when performing the comparison among pigs fed MT and CON. Even though, despite the reduction in feed intake in pigs fed MT+D in comparison to pigs fed MT during week 1, pigs fed MT+D tended to have increased averaged daily gain than pigs fed MT during week 4. The improvement observed in growth performance in pigs challenged with deoxynivalenol could be due to the multiple component-approach (activated aluminosilicate, autolyzed yeast, probiotic yeast culture, calcarium marine algae powder, and enzyme complex) in the mycotoxin-detoxifying additive, as seen that deoxynivalenol is a mycotoxin that is known to be adsorbed or neutralized with difficulty [14].

The aluminosilicates have proven ability to adsorb mycotoxins, even though the deoxynivalenol adsorption is not as significant as in comparison to other mycotoxins [13,15]. The activated aluminosilicate, known as bentonite, was used in the current study to adsorb deoxynivalenol. As reviewed by Chaytor et al. [14], the negative charge of AlO_4 is responsible for the adsorbing ability towards mycotoxins, but as dependent on electrical interaction the adsorption of deoxynivalenol (non-polar) is not as substantial as for aflatoxins (polar). Therefore, mycotoxin detoxifiers targeting deoxynivalenol often show higher detoxifying capacity if multiple components are present both *in vitro* and *in vivo* [12,22].

The autolyzed yeast, used as a source of yeast cell wall, is the second form of adsorbent present in the composition of the mycotoxin-detoxifying additive. It differs from activated aluminosilicates for being organic, whereas activated aluminosilicates are inorganic adsorbents. Yeast- and algae-derived β-glucans can show higher deoxynivalenol adsorbing abilities than mineral adsorbents [15]. The β-glucans from the yeast cell wall may present around 7-times more adsorbing capacity than bentonites, being the highest in comparison to other organic adsorbents, like cellulose or a mixture of plan derivatives, microorganisms, and minerals [13].

The third ingredient in the mycotoxin-detoxifying additive is the probiotic yeast culture of *Saccharomyces cerevisiae* at 2×10^7 CFU/kg. The microbiota is able of microbial detoxification towards deoxynivalenol, but pig microbiota seems to have a lower detoxifying ability in comparison to ruminants and chickens, as observed *in vitro* [36]. Another *in vitro* study showed that pig microbiota from duodenum and jejunum have minimal detoxifying properties, whereas deoxynivalenol detoxification was observed for the microbiota from the cecum, colon, and rectum [37]. Nevertheless, deoxynivalenol is absorbed almost in its entirety in the stomach and proximal small intestine of the pig [38]. Consistently, during 24 hours after deoxynivalenol

administration to pigs, either intravenous or intragastrical, showed that more than 90% of the initial dose was excreted without modifications [38]. Such findings may suggest that pigs are more vulnerable to deoxynivalenol in comparison to other species because of the rapid absorption of deoxynivalenol in the proximal gastrointestinal tract and its limited metabolism of the mycotoxin. The gastrointestinal microbiome of pigs may be modified when feeding deoxynivalenol contaminated diets [39], indicating that the gastrointestinal microbiome can be modulated to avoid deoxynivalenol toxicity. Thus, pig tolerance to deoxynivalenol can be improved by probiotic with deoxynivalenol-detoxifying properties, contained in the mycotoxin-detoxifying additive, to enhance microbial detoxification by promoting beneficial species. The microbial detoxification of deoxynivalenol is described to be mainly performed by anaerobic gram-positive bacteria in the gastrointestinal tract by conversion to the de-epoxide form [40]. Microbes with deoxynivalenol-detoxifying capacity are Eubacteria, *Anaerofilum*, *Collinsella*, *Bacillus*, and Clostridiales [40]. The use of yeast in the livestock feed chain [16], directed scientific efforts for its further use in livestock feed for mycotoxin detoxification. Under deoxynivalenol challenge, *S. cerevisiae* is able to reduce inflammation and oxidative stress and to increase cell survivability [17,41].

Modulating the gastrointestinal environment may help boost the adsorbing and detoxification properties of the mycotoxin-detoxifying additive in deoxynivalenol challenged pigs. The calcarium marine algae powder in the mycotoxin-detoxifying additive is derived from *Lithothamnium calcareum*. *In vitro*, *L. calcareum* has shown adsorption ability of zearalenone both in acidic and neutral pH [20]. In broilers challenged with mycotoxin diet, the addition of *L. calcareum* could recover animal weight gain [42] and prevent aflatoxicosis [21]. But there is a

lack of studies regarding the adsorbability towards deoxynivalenol, especially concerning *in vivo* studies in pigs.

The enzymes present in the mycotoxin-detoxifying additive associated with the probiotic (*S. cerevisiae*) claim to have the ability to neutralize mycotoxins. The enzyme complex included in the mycotoxin-detoxifying additive formulation is composed of esterase, epoxide-reductase, and peptidase. The esterase activity has two main targets. First, as a detoxifier of zearalenone and fumonisins, which often are co-contaminants in animal feedstuff and feed due to being originated from *Fusarium* fungi [1]. As reviewed by Loi et al. [19] the esterase has detoxifying activity by cleaving the lactone ring in zearalenone structure or breaking down the diester bonds in fumonisin B1. In addition, the reaction catalyzed by the esterase serves as an initial step for deoxynivalenol de-epoxidation. The deoxynivalenol in its masked naturally occurring forms, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, are converted to deoxynivalenol by the esterase activity [43]. The epoxide-reductase is responsible for the deoxynivalenol conversion to de-epoxy-deoxynivalenol, a non-toxic metabolite [18]. Besides, the epoxide-reductase activity relies on energy [36] or protein and amino acid [44] availability to perform the de-epoxidation reaction. Thus, the peptidase in the mycotoxin-detoxifying additive may provide the source of protein and amino acids required to enhance the epoxide-reductase activity. The slight improvement in averaged daily gain proportioned by feeding MT+D diet may indicate the detoxifying properties of the mycotoxin-detoxifying additive when included at 0.2% in diets contaminated with 3.1 mg/kg of deoxynivalenol. Comparable results were observed in growing pigs challenged with deoxynivalenol and zearalenone, where inclusion of a similar enzyme blend could enhance the growth performance of pigs [45].

In the current study, pigs fed MT had lower blood urea nitrogen/creatinine and AST/ALT than pigs fed CON, whereas pigs fed MT+D showed a recovery by increased AST/ALT than pigs fed MT. Likewise, pigs challenged with deoxynivalenol showed decreased blood urea nitrogen/creatinine and a mycotoxin detoxifier (composed of a mixture of clay, inactivated *S. cerevisiae* and its fermentation extracts, antioxidant, and botanicals) also caused an increase in AST/ALT in comparison to pigs fed diet contaminated with deoxynivalenol [24]. However, mice challenged with deoxynivalenol and zearalenone showed increased blood urea nitrogen due to mycotoxin impairment over kidney function [46]. Such outcomes may suggest that kidney damage was not significant in the current trial to lead to increased blood urea nitrogen. Instead, the deoxynivalenol inhibitory effect over protein synthesis, known as ribotoxic stress [4], could have a role in reducing protein metabolism and, thus, reducing the synthesis of aspartate aminotransferase and circulating levels of urea nitrogen [47].

The site of intestinal tissue collection was grounded on the fact that deoxynivalenol is mostly absorbed in the proximal gastrointestinal tract [38]. In addition, pigs fed deoxynivalenol presented a similar reduction on feed intake but had a greater reduction in body weight gain in comparison to pigs administered deoxynivalenol intraperitoneally [48], suggesting that deoxynivalenol may have local effects at the gastrointestinal tract that prevents nutrient digestibility and/or absorption. Indeed, the decrease in serum glucose observed in pigs fed MT in comparison to CON may be due to deoxynivalenol inhibition over SGLT1 in the brush border membrane in the small intestine, which limits glucose absorption. The deoxynivalenol-induced decrease in glucose uptake was demonstrated to be caused by impairing the expression and functions of the SGLT1 [49].

The lower proliferative rate in mid-jejunum observed in pigs fed MT+D in comparison to pigs fed MT in measuring Ki-67 positive cells proportion might suggest that MT+D diet could reduce deoxynivalenol toxicity in enterocytes and eventually reduce enterocyte death. Deoxynivalenol challenge causes increased expression of pro-inflammatory cytokines, such as IL-8, triggering cell apoptosis in the pig intestine [17,22]. Using *S. cerevisiae* as a probiotic source *in vitro* has shown the ability to reduce the expression of IL-8, TNF-α, and programmed cell death-related genes [41,50]. In a study with pig intestinal explants, the probiotic use of *S. cerevisiae* could counteract deoxynivalenol-induced inflammation and oxidative stress [17]. But there was no difference observed in inflammatory or immune markers measured in mid-jejunum in the current study. The only variable altered was total glutathione. The lower total glutathione presented by pigs fed MT in comparison to pigs fed CON may indicate that the concentration of 3.1 mg/kg of deoxynivalenol was surpassing the antioxidative capacity of pig mid-jejunum, depleting glutathione cell supply. Deoxynivalenol has inhibitory action over the vitamin C pathway, resulting in oxidative stress in pig intestine [17]. Vitamin C is important in glutathione regeneration to prevent oxidative stress, which may result in cell death [51,52].

4.6. Conclusion

In conclusion, feeding supplemental 1.9 mg/kg of deoxynivalenol reduced the average daily gain and debilitated hepatic and gut health by leakage of hepatic enzymes, impaired nitrogen metabolism, reducing glucose supply to the organism, and increasing oxidative stress in the gut. The mycotoxin-detoxifying additive mainly composed of bentonite (as adsorbent), calcarium algae powder (as adsorbent), enzymes (as mycotoxin inactivators), and *Saccharomyces cerevisiae* (as a source of β-glucans – adsorbent and immune modulator – and as

probiotic) enhanced hepatic health, glucose levels, and reduced proliferation of enterocytes in the crypt indicating attenuated gut damage in pigs fed diets contaminated with deoxynivalenol. However, the improvements promoted by the mycotoxin-detoxifying additive shown in gut health did not result in improved growth performance.

4.7. References

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CHAPTER 5. ASSESSMENT OF EFFECTS OF WEANING WEIGHT ON GROWTH PERFORMANCE AND HEALTH OF NEWLY-WEANED PIGS UNDER MYCOTOXIN CHALLENGE

5.1. Abstract

This study investigated the effects of mycotoxin challenge on the growth and health of pigs with different weaning weights. Before allotment, 10 out of 106 weanling pigs were euthanized to collect intestine and mucosal-associated microbiome. Ninety-six weanling pigs were assigned following an RCBD in a 2 x 2 factorial arrangement with 3 pigs per pen on d 0. Factors were: weaning weight, light (< 7.5 kg) or heavy (> 9.0 kg); and dietary mycotoxin, low or high (additional: 0.2 mg/kg aflatoxins, 2.0 mg/kg deoxynivalenol). Fecal score and growth performance were recorded until pigs achieved 20 kg. At 20kg, pigs were sampled for blood, ileal digesta, intestine, and mucosal-associated microbiome. Data were analyzed using SAS mixed procedure. On d 0, light pigs had decreased intestinal interleukin-8 ($p < 0.05$), increased ($p < 0.05$) TNF-α, and increased ($p < 0.05$) α-diversity indexes of mucosal-associated microbiome. Overall, light pigs had decreased ($p < 0.05$) BW, ADG, ADFI, and G:F. Mycotoxins decreased ($p < 0.05$) BW, ADG, ADFI, and G:F. Light pigs tended to have increased fecal score on d 0 ($p = 0.080$), d 10 ($p = 0.069$), and increased ($p < 0.05$) on d 20. At 20 kg, mycotoxins decreased AID of nitrogen ($p < 0.05$). Light pigs had increased ($p < 0.05$) intestinal malondialdehydes and interleukin-8. Mycotoxins tended to increase ($p = 0.060$) intestinal TNF-α. There was no difference in the microbiome profile of light pigs in comparison to heavy pigs at 20 kg. In conclusion, light pigs were more susceptible to inflammation and had impaired intestinal health due to weaning stress, whereas mycotoxins impaired the health and growth of pigs regardless of weaning weight.

Keywords: aflatoxin; deoxynivalenol; weaning age; weaning stress

5.2. Introduction

Weaning events such as, change of diet, facilities, and pig-to-pig aggression are among the factors that cause weaning stress. The weaning stress can lead to inflammatory activation in the intestine and damage of enterocytes [1] reducing feed digestibility and nutrient absorption. It has long been demonstrated that pigs weaned older are less susceptible to weaning stress and, thus, have improved feed intake and body weight gain [2]. More recently, weaning stress was linked to long-lasting or permanent detrimental effects on the gastrointestinal tract and the immune system of pigs [3]. Also, weaning weight, as an estimate of weaning age [4], can affect the pig intestinal microbiome [5] as well as health and growth during the nursery period [3].

Aflatoxins and deoxynivalenol are mycotoxins produced by filamentous fungi which may potentially contaminate feed and cause detrimental effects in pigs. Aflatoxins and deoxynivalenol impair protein synthesis [6,7], cause oxidative stress [8,9], diminish the expression and activity of nutrient transporters in the brush border membrane of enterocytes [10,11], and alter intestinal microbiome profile [12]. Thus, pigs challenged with mycotoxins have impaired intestinal health, reduced digestibility of nutrients in feed [13,14], hepatic damage [15], resulting in reduced growth performance [13,14,16].

Pig susceptibility to the toxic effects of mycotoxins was previously shown to decrease with age [16]. Therefore, the current study investigated the effects of supplemental dietary 0.2 mg/kg of aflatoxins and 2.0 mg/kg of deoxynivalenol on growth performance and health of newly-weaned pigs with different weaning weights.

5.3. Materials and Methods

The experiment was carried out at North Carolina State University (Raleigh, NC, USA) in the Swine Evaluation Station (Clayton, NC, USA). The protocol for this experiment was approved by the Institutional Animal Care and Use Committee at North Carolina State University.

5.3.1. Animals and Diets

A hundred and eighty-six newly-weaned pigs (21 to 35 d of age) were moved to the research farm right after weaning. Upon receiving, one hundred and six pigs were divided into 2 groups based on body weight: (i) L, light pigs with weaning weight equal or lower to 7.5 kg; (ii) H, heavy pigs with body weight equal or above 9.0 kg. Within each weaning weight group, pigs were ranked by body weight into three tiers (high, intermediate, and low weaning weight). Subjects with the highest (n = 2), intermediate (n = 2), and lowest (n = 1) weaning weights within L and H groups were selected (totaling n = 10). The selected pigs were euthanized by desensitization by captive bolt followed by vena cava section for exsanguination for sample collection. The remaining ninety-six pigs were assigned following a randomized complete block design (using sex as a block) to 4 treatments based on a 2 x 2 factorial arrangement with three pigs per pen, totaling 32 pens, 16 replicates per factor, and 8 replicates per treatment. Body weight factor: either (i) L, light pigs with weaning weight equal or lower to 7.5 kg; (ii) H, heavy pigs with body weight equal or above 9.0 kg. Dietary factor: (i) "-",diet with low mycotoxin concentration; (ii) "+", diet with high mycotoxin concentration (additional 2 mg/kg of deoxynivalenol and 0.18 mg/kg of aflatoxins). Additional mycotoxin contamination was achieved by the inclusion of deoxynivalenol-contaminated corn DDGS and aflatoxin-contaminated corn. Pigs were fed phase 1 diets from the beginning of the trial (d 0) until

achieving 11 kg of body weight and phase 2 diets from 11 to 20 kg of body weight. Diets were formulated to meet or exceed NRC [17] requirements for nursery pigs (Table 1). At the end of phase 2, within each sex and dietary treatment, one pig per pen with the median body weight was selected from the heavier, intermediate, and lighter pens. The 24 selected pigs were sampled after euthanasia following the same procedure as described previously for sample collection on d 0.

Table 1. Experimental diets formulated for newly-weaned pigs with different weaning weights and fed diets with (+) or without (-) additional mycotoxins.

Dietary phase	**1**		**2**	
Mycotoxins	**-**	+	**-**	+
Ingredient, %				
Ground corn	29.11	23.11	40.87	34.84
AF corn	0.00	6.00	0.00	6.00
Corn DDGS	22.00	0.00	22.00	0.00
DON corn DDGS	0.00	22.00	0.00	22.00
Soybean meal	15.56	15.56	26.50	26.50
Whey permeate	20.00	20.00	5.00	5.00
Poultry meal	4.00	4.00	0.00	0.00
Blood plasma	5.00	5.00	0.00	0.00
Limestone	1.19	1.19	1.10	1.10
Dicalcium phosphate	0.08	0.08	0.60	0.60
L-lysine HCl	0.48	0.48	0.42	0.42
DL-methionine	0.11	0.11	0.07	0.07
L-threonine	0.07	0.07	0.07	0.07
Salt	0.22	0.22	0.22	0.22
Mineral supplement	0.15	0.15	0.15	0.15
Vitamin supplement	0.03	0.03	0.03	0.03
Fat	2.00	2.00	2.50	2.50
Titanium dioxide	0.00	0.00	0.5[1]	0.5[1]
Calculated composition				
ME, kcal/kg	3,423	3,423	3,397	3,397
SID Lys, %	1.35	1.35	1.23	1.23
Ca, %	0.80	0.80	0.70	0.70
Aflatoxins, mg/kg	0.00	0.18	0.00	0.18
Deoxynivalenol, mg/kg	0.00	2.00	0.00	2.00

AF corn, corn contaminated with aflatoxins at 3 mg/kg. Corn DDGS, corn distillers dried grains with solubles. DON corn DDGS, corn DDGS contaminated with deoxynivalenol.

[1], Titanium dioxide was added at 0.5% to phase 2 diets as an external marker for assessment of apparent ileal digestibility of nutrients in feed.

5.3.2. Sample Collection and Processing

Feed samples were randomly collected from each dietary treatment and phase totaling 2 kg each. Subsamples of 300 g were sent for proximate analysis at the North Carolina Department of Agriculture (Raleigh, NC, USA). Subsamples of 2 g were ground and sent to the Veterinary Diagnostic Laboratory (North Dakota State University, Fargo, ND, USA) for determination of concentrations of mycotoxins by liquid chromatography-tandem mass spectrometry.

The fecal score was assessed by observing feces on the floor according to a scale ranging from 1 to 5 [18] in the morning (0700 h) of d 0, 3, 5, 7, 10, 15, and 20. Pens were washed with a hose in the afternoon before of each fecal score assessment.

The 10 pigs euthanized at the beginning of the study and the 24 pigs euthanized at the end of the study were sampled for tissue and mucosa, from the duodenum and proximal jejunum [19]. One 5 cm-long section of each, duodenal and jejunal tissues, was fixed in buffered formalin at room temperature for 72 hours. After that, 2 cross-sections of 0.5 cm of each sample were moved to cassettes and transferred to 70% ethanol. The cross-sections were sent to the North Carolina State University Histopathology Laboratory (College of Veterinary Medicine, Raleigh, NC, USA) for Ki-67 staining according to procedures described by Kim et al. [16]. Intestinal mucosa samples were obtained by scraping intestinal sections of 15 cm and were placed in vials that were immediately immersed in liquid nitrogen for further assessment of immune and oxidative stress markers. One more vial of proximal jejunal mucosa was obtained for microbiome sequencing and placed in liquid nitrogen. The vials were transferred, by the end of the day, to a -80 °C freezer. Duodenum and proximal jejunum were chosen as the sampling sites because deoxynivalenol is readily absorbed by paracellular diffusion and the proximal small intestine seems to be more affected in pigs fed diets with deoxynivalenol [13].

The 24 pigs chosen to be sampled at the end of the study were also sampled for blood and ileal digesta. Two days prior to pigs achieving 20 kg of body weight, 10 mL of blood was collected in tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) by puncturing the jugular vein (0.8 x 32 mm needles, Eclipse, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) for performing serum analyses of biochemical variables and electrolytes. Blood samples were allowed to clot for 4 h before centrifuging at 1,509 × g at 4 °C for 15 minutes (5811F, Eppendorf, Hamburg, HH, Germany). The supernatant, blood serum, was transferred to duplicates of 1.5 mL vials (Fisherbrand, Fisher Scientific, Hampton, NH, USA) and stored at -80 °C freezer (812660-760, Thermo Fisher Scientific, Waltham, MA, USA) until laboratory analyses. The ileal digesta was collected by gradually squeezing the ileal content delimited proximally by the ileocecal fold and caudally by the ileocecal junction. Ileal digesta containers were immediately immersed in ice and then stored at -20 ºC until further analyses to estimate the ileal digestibility of dry matter, protein, gross energy, and ether extract contents in the feed.

5.3.3. Assay Procedures

Histology sections stained for Ki-67 antigen were measured for villus height (from the tip of the villus to the villus-crypt junction), villus width (perpendicular line to the longer axis of the villus at one-half of the villus height), and crypt depth (from villus junction to the base of the crypt) were taken from well-oriented intact villus and its associated crypt [9]. The average among 10 measurements was used for each pig. The estimation of the proliferative rate was obtained by calculating the proportion of Ki-67-positive cells to the total cell number in the crypt [9] by using ImageJS software [20]. One same evaluator executed all the histological analyses for intestinal morphology and Ki-67 counting.

One vial of duodenal and proximal jejunal mucosa was used for assessing immune and oxidative stress markers in each intestinal section. The markers were evaluated by quantifying protein carbonyls (STA-310, Cell Biolabs, Inc., San Diego, CA, USA), malondialdehydes (STA-330, Cell Biolabs, Inc., San Diego, CA, USA), tumor necrosis factor-alpha (PTA00, R&D Systems, Inc., Minneapolis, MN, USA), interleukin-8 (P8000, R&D Systems, Inc., Minneapolis, MN, USA), immunoglobulin A (E100-102, Bethyl Laboratories, Inc., Montgomery, TX, USA), and immunoglobulin G (E100-104, Bethyl Laboratories, Inc., Montgomery, TX, USA) relative to the protein content of samples (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). First, cellular content was extracted by thawing vials on ice and homogenizing (Tissuemiser, Thermo Fisher Scientific, Waltham, MA, USA) 1 g of the sample with 2 mL of PBS (MP Biomedicals, Inc., Santa Ana, CA, USA) for 30 seconds. The supernatant obtained after centrifuging at 87,000 × g for 20 min were placed in individual vials to be used for measuring each of the markers and stored at −80 ◦C until further use. The manufacturer's manual for each kit was followed in the laboratory assays as described by Holanda et al. [13].

The second vial of proximal jejunal mucosa was used to assess the mucosa-associated microbiome. The DNA was extracted from mucosa with QIAGEN's QIAamp® DNA Stool MiniKit (Qiagen, Crawley, SXW, UK). The DNA samples were sent to Mako Medical Laboratories (Raleigh, NC, USA) for microbial sequencing using the 16S rDNA technique. The Ion Chef instrument was used to prepare the samples for template and sequencing was performed on the Ion S5 system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Variable regions V1, V2, V3, V4, V6, V7, V8, and V9 of the 16S rRNA gene were amplified with the Ion 16S Metagenomics Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The hypervariable regions were processed using the Torrent Suite Software (version 5.2.2; Thermo Fisher

Scientific, Inc., Waltham, MA, USA) to produce raw unaligned sequence data files for further analysis. Sequence data analysis, alignment to GreenGenes and MicroSeq databases, alpha diversity plot generation, and OTU table generation were performed by the Ion Reporter Software Suite of bioinformatics analysis tools (version 5.2.2; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples were analyzed using Ion Reporter's Metagenomics 16S workflow powered by Qiime (Quantitative Insights Into Microbial Ecology, version w1.1). Alpha-diversity was estimated with 3 indexes: Chao1, Shannon-Weaver, and Simpson.

Serum samples were submitted for biochemical profiling at ANTECH Diagnostic Laboratory (Cary, NC, USA).

Apparent ileal digestibility of dry matter [21], protein (828 Series Carbon/Nitrogen Analyzer with Cornerstone Brand Software, FP828, Model Number 622-100-700; LECO, St. Joseph, MI, USA), gross energy (6200 Calorimeter, Parr Instrument Company, Moline, IL, USA), and ether extract (method 920.39, [22]) contents in feed using titanium dioxide as an external marker.

5.3.4. Statistical Methods

The use of phases based on pig body weight was based on previous studies [23–26] as well as to enable the comparison of the body weight factor at different time points during the study.

For the statistical analysis, SAS 9.3 software (Cary, NC, USA) was used to process data with factors and their interaction as fixed effects and sex block as a random effect using the MIXED procedure. The pen was considered the experimental unit for growth performance and fecal score data. The pig, representing its pen, was considered the experimental unit for all other

variables. Results were considered statistically different for $p < 0.05$ and considered a tendency for $0.05 \leq p < 0.10$.

5.4. Results

In the finished diets, the average concentration of mycotoxins in the low (-) and high (+) mycotoxin diet for aflatoxins was 0.02 and 0.21 mg/kg and for deoxynivalenol was 0.52 and 2.32 mg/kg, respectively (Table 2). The resultant mycotoxin concentrations for additional aflatoxins and deoxynivalenol are close to the expected concentrations, an additional 0.19 mg/kg of aflatoxins and an additional 1.8 mg/kg of deoxynivalenol. Of note, two pigs were removed from the study, one on d 11, due to neurological signs, and the other on d 18, because of severe diarrhea. Both were light pigs, the first was receiving the diet high in mycotoxins and the second was not.

Table 2. Concentrations of mycotoxins in finished diets formulated for newly-weaned pigs based on a 2-phase feeding program.

Dietary phase	**1**		**2**	
Mycotoxins	**-**	+	-	+
Aflatoxin B1, mg/kg	< 0.020	0.130	< 0.020	0.242
Aflatoxin B2, mg/kg	< 0.020	< 0.020	< 0.020	0.033
Deoxynivalenol, mg/kg	0.472	2.258	0.566	2.389
Fumonisin B1, mg/kg	0.326	1.810	0.598	2.000
Fumonisin B2, mg/kg	< 0.200	0.378	< 0.200	0.445
Zearalenone, mg/kg	< 0.100	0.159	< 0.100	0.171

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). Concentrations determined by mycotoxin screen by liquid chromatography-tandem mass spectrometry (Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, USA).

Light pigs had lower ($p < 0.05$) body weight on d 0, as expected, and by the end of phase 1 (Table 3). Pigs fed high mycotoxins had lower ($p < 0.05$) body weight by the end of phase 2. Among light pigs feeding high mycotoxins tended to result in lower ($p = 0.070$) body weight on d 31. Light pigs had lower ($p < 0.05$) average daily gain (ADG) during phase 1 but higher ADG during phase 2 than heavy pigs. Light pigs tended to have lower ($p = 0.054$) ADG in the overall period than heavy pigs. Pigs fed high mycotoxins had lower ($p < 0.05$) ADG during phase 2 and in the overall period. Light pigs had lower ($p < 0.05$) average daily feed intake (ADFI) during phase 1 and in the overall period, but higher ($p < 0.05$) ADFI during phase 2 than heavy pigs. Pigs fed high mycotoxins had lower ($p < 0.05$) ADFI during phase 2 and in the overall period. Pigs fed high mycotoxins tended to have a higher ($p = 0.055$) gain to feed ratio (G/F) in the overall period.

Table 3. Animal performance variables observed in weanling pigs with different weaning weights and consuming diets with low (-) or high (+) mycotoxins.

Body weight (BW)	**< 7.5 kg**		**> 9.0 kg**		**SEM**	***p* Value**		
Mycotoxins (MTX)	**-**	**+**	**-**	**+**		**BW**	**MTX**	**BW vs. MTX**
Body weight, kg								
d 0	6.9	6.9	9.8	9.8	0.2	<0.001	0.974	0.961
Phase 1	10.5	10.1	11.3	11.1	0.2	0.029	0.435	0.756
Phase 2	22.4	19.8	21.2	19.7	0.8	0.422	0.015	0.508
ADG, g								
Phase 1	257	228	371	326	32	0.002	0.259	0.800
Phase 2	686	572	584	508	34	0.006	0.002	0.524
Overall	498	416	544	473	30	0.054	0.006	0.836
ADFI, g								
Phase 1	373	316	532	498	32	<0.001	0.168	0.727
Phase 2	1,038	807	842	719	50	<0.001	<0.001	0.129
Overall	737	585	783	677	38	0.033	<0.001	0.452
G/F								
Phase 1	0.70	0.70	0.70	0.66	0.05	0.639	0.615	0.648
Phase 2	0.67	0.71	0.69	0.71	0.02	0.687	0.119	0.444
Overall	0.67	0.71	0.68	0.70	0.01	0.945	0.055	0.469

ADG, average daily gain; ADFI, average daily feed intake; G/F, gain to feed ratio. Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups).

Regarding the fecal score, light pigs tended to present higher fecal score on d 0 (p = 0.080) and d 10 ($p = 0.069$; Table 4). On d 15, there was a tendency for interaction ($p = 0.092$) where light pigs fed high mycotoxins tended to have higher ($p = 0.096$) fecal score than heavy pigs fed diets with low mycotoxins. Light pigs presented higher ($p < 0.05$) fecal score on d 20. There was no difference in the fecal score on d 3, 5, or 7 among pigs.

Table 4. Fecal score observed in weanling pigs with different weaning weights and consuming diets with or without mycotoxins.

Body weight (BW)	< 7.5 kg		> 9.0 kg		SEM	*p* Value		
Mycotoxins (MTX)	-	+	-	+		BW	MTX	BW vs. MTX
d 0	2.25	2.25	1.63	1.13	0.48	0.080	0.607	0.607
d 3	2.25	2.25	1.88	1.75	0.41	0.294	0.880	0.880
d 5	2.50	3.13	2.75	2.75	0.43	0.886	0.476	0.476
d 7	2.13	2.38	2.25	2.38	0.54	0.909	0.733	0.909
d 10	2.25	2.50	1.25	2.00	0.40	0.069	0.217	0.532
d 15	2.63^{a}	1.88^{aX}	1.00^{bY}	1.50^{b}	0.36	0.010	0.730	0.092
d 20	1.88	1.63	1.38	1.00	0.29	0.020	0.179	0.785

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). ^{a,b} Means within a row with a different superscript differ ($p < 0.05$). ^{X,Y} Means within a row with a different superscript tend to differ ($0.05 \leq p < 0.10$).

Pigs fed high mycotoxins tended to have lower ($p = 0.078$) dry matter apparent ileal digestibility (Table 5). Pigs fed high mycotoxins had lower ($p < 0.05$) nitrogen apparent ileal digestibility. There was a tendency for interaction ($p = 0.053$) where among light pigs feeding high mycotoxins increased ($p < 0.05$) ether extract apparent ileal digestibility, whereas there was no mycotoxin effect among heavy pigs. There was no difference in gross energy apparent ileal digestibility among pigs.

Table 5. Apparent ileal digestibility in weanling pigs with different weaning weights and consuming diets with or without mycotoxins at 20 kg of body weight.

Body weight (BW)	**< 7.5 kg**		**> 9.0 kg**		**SEM**	***p* Value**		
Mycotoxins (MTX)	**-**	**+**	**-**	**+**		**BW**	**MTX**	**BW x MTX**
Dry matter, %	65.9	62.1	67.2	58.8	3.3	0.768	0.078	0.483
Gross energy, %	66.1	63.5	67.3	59.3	3.3	0.661	0.126	0.425
Nitrogen, %	79.2	69.7	79.0	68.5	2.8	0.813	0.002	0.858
Ether extract, %	73.0^{b}	87.3^{a}	77.9^{ab}	74.5^{b}	4.3	0.370	0.215	0.053

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). [a,b] Means within a row with a different superscript differ ($p < 0.05$).

Light pigs had higher ($p < 0.05$) blood serum ALP and lower ($p < 0.05$) chloride (Table 6). Pigs fed high mycotoxins had reduced ($p < 0.05$) albumin and albumin to globulin ratio. There was a tendency for interaction ($p = 0.091$) where among light pigs feeding high mycotoxins increased globulin, whereas there was no difference among heavy pigs. There was an interaction ($p < 0.05$) where there was no difference among light pigs, whereas among heavy pigs feeding high mycotoxins tended to increase ($p = 0.093$) creatinine. There was a tendency for interaction ($p = 0.053$) where among heavy pigs feeding high mycotoxins increased ($p < 0.05$) BUN to creatinine ratio, whereas there was no difference among light pigs. There was an interaction ($p < 0.05$) where among heavy pigs feeding high mycotoxins decreased ($p < 0.05$) phosphorus, whereas there was no difference among light pigs. There was no difference observed for other blood variables among pigs.

Table 6. Serum variables observed for liver health and electrolytes in weanling pigs with different weaning weights and consuming diets with or without mycotoxins at 20 kg of body weight.

Body weight (BW)	< 7.5 kg		> 9.0 kg		SEM	*p* Value		
Mycotoxins (MTX)	-	+	-	+		BW	MTX	BW x MTX
Liver health								
Total protein, g/dL	5.05	5.08	5.07	4.88	0.15	0.496	0.576	0.422
Albumin, g/dL	3.28	2.95	3.07	2.93	0.14	0.311	0.051	0.383
Globulin, g/dL	1.77^{b}	2.13^{a}	2.00^{ab}	1.95^{ab}	0.12	0.833	0.192	0.091
Albumin/Globulin	1.88	1.43	1.57	1.52	0.12	0.340	0.050	0.110
AST, IU/L	34.8	32.2	35.2	41.2	5.3	0.386	0.755	0.420
ALT, IU/L	19.3	17.5	19.7	18.7	1.4	0.587	0.310	0.762
AST/ALT	1.80	1.90	1.81	2.16	0.25	0.587	0.370	0.616
ALP, IU/L	277	263	204	241	23	0.049	0.635	0.275
CPK, IU/L	884	884	849	910	244	0.985	0.901	0.903
Cholesterol, mg/dL	81.5	82.0	76.2	74.0	5.6	0.194	0.868	0.791
BUN, mg/dL	12.0	11.7	12.7	10.7	0.9	0.861	0.230	0.386
Creatinine, mg/dL	0.63^{b}	0.58^{b}	0.65^{abY}	0.72^{aX}	0.03	0.011	0.758	0.041
BUN/Creatinine	19.0^{a}	20.2^{a}	19.3^{a}	14.8^{b}	1.4	0.085	0.240	0.053
Glucose, mg/dL	113	111	109	102	5	0.166	0.410	0.653
Electrolytes								
Phosphorus, mg/dL	9.17^{ab}	9.78^{a}	9.78^{a}	8.67^{b}	0.34	0.470	0.470	0.019
Calcium, mg/dL	10.8	10.8	10.7	10.5	0.2	0.339	0.705	0.644
Sodium, mEq/L	145	145	145	145	1	0.445	0.445	0.645
Potassium, mEq/L	5.52	5.45	5.77	5.48	0.28	0.466	0.369	0.576
Na/K	26.3	26.8	25.2	26.8	1.3	0.528	0.247	0.528
Chloride, mEq/L	102	102	105	105	1	0.007	0.911	0.911

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; CPK, creatine phosphokinase. [a, b, c] Means within a row with a different superscript differ ($p < 0.05$). [X, Y] Means within a row with a different superscript tend to differ ($0.05 \leq p < 0.10$).

On d 0, light pigs tended to have higher ($p = 0.085$) malondialdehydes in duodenal mucosa and had higher ($p < 0.05$) malondialdehydes in jejunal mucosa (Table 7). Light pigs had lower ($p < 0.05$) interleukin 8 in duodenal mucosa but higher ($p < 0.05$) interleukin 8 in jejunum

mucosa. Light pigs had higher ($p < 0.05$) immunoglobulin G in duodenal and jejunal mucosa. Light pigs had higher ($p < 0.05$) protein carbonyls in jejunal mucosa. Light pigs tended to have higher ($p = 0.055$) tumor necrosis factor-α but tended to have lower ($p = 0.090$) immunoglobulin A.

Table 7. Immune response and oxidative stress markers, and histometric measurement from duodenum and proximal jejunum in pigs with different weaning weights upon weaning.

Body weight	< 7.5 kg	> 9.0 kg	SEM	*p* Value
Duodenum				
MDA, μM/mg of protein	1.137	0.629	0.229	0.085
Protein carbonyl nmol/mg of protein	3.029	2.563	0.304	0.315
TNF-α, pg/mg of protein	1.714	1.281	0.180	0.132
IL-8, ng/mg of protein	0.641	2.209	0.343	<0.001
IgA, pg/mg of protein	5.115	5.377	1.140	0.860
IgG, pg/mg of protein	10.448	4.577	1.391	0.020
Villus width, μm	151	167	6	0.089
Villus height (V), μm	288	297	16	0.677
Crypt depth (C), μm	290	302	31	0.789
V:C	1.04	1.00	0.09	0.814
Ki-67, %	22.7	25.5	4.0	0.411
Jejunum				
MDA, μM/mg of protein	1.194	0.578	0.154	0.025
Protein carbonyl nmol/mg of protein	3.236	2.628	0.163	0.034
TNF-α, pg/mg of protein	1.594	0.951	0.198	0.055
IL-8, ng/mg of protein	1.024	1.636	0.211	0.013
IgA, pg/mg of protein	2.469	3.871	0.874	0.090
IgG, pg/mg of protein	12.017	5.072	1.527	0.015
Villus width, μm	121	153	9	0.038
Villus height (V), μm	171	277	8	<0.001
Crypt depth (C), μm	180	247	14	0.014
V:C	0.97	1.12	0.05	0.059
Ki-67, %	36.2	27.9	3.6	0.154

MDA, malondialdehydes; TNF-α, tumor necrosis factor-alpha; IL-8, interleukin 8; IgA, immunoglobulin A; IgG, immunoglobulin G. Ki-67 is an estimate of the proliferative rate, calculated based on the proportion of nuclei positive to Ki-67 staining to the total nucleus number. Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups).

Regarding intestinal histomorphology measurements on d 0, light pigs tended to have lower ($p = 0.089$) villus width in the duodenum and had lower ($p < 0.05$) villus width in the

proximal jejunum. Light pigs had lower ($p < 0.05$) villus height, crypt depth, and tended to have lower ($p = 0.059$) villus height to crypt depth ratio. At 20 kg of body weight, there was no difference among histomorphometry variables in the duodenum nor the jejunum, except for crypt depth in the proximal jejunum. Light pigs tended to have higher ($p = 0.053$) crypt depth.

At 20 kg of body weight, light pigs had lower ($p < 0.05$) malondialdehydes in duodenal mucosa but higher ($p < 0.05$) malondialdehydes in proximal jejunal mucosa (Table 8). Pigs fed high mycotoxins had higher ($p < 0.05$) protein carbonyls in duodenal mucosa and tended to have higher ($p = 0.080$) protein carbonyls in proximal jejunal mucosa. Also, light pigs had higher ($p < 0.05$) protein carbonyls in proximal jejunal mucosa. There was an interaction ($p < 0.05$) for tumor necrosis factor-α in proximal jejunal mucosa, where among light pigs feeding high mycotoxins increased ($p < 0.05$) tumor necrosis factor-α concentration per mg of protein, whereas there was no difference among heavy pigs. Light pigs had higher ($p < 0.05$) interleukin 8 and tended to have higher ($p = 0.055$) immunoglobulin A in proximal jejunal mucosa.

Table 8. Immune response and oxidative stress markers, and histometric measurement from duodenum and proximal jejunum in pigs with different weaning weights and consuming diets with or without mycotoxins at 20 kg of body weight.

Body weight (BW)	**< 7.5 kg**		**> 9.0 kg**		**SEM**	***p* Value**		
Mycotoxins (MTX)	**-**	**+**	**-**	**+**		**BW**	**MTX**	**BW x MTX**
Duodenum								
MDA, µM/mg of protein	0.861	0.844	1.496	1.082	0.210	0.049	0.314	0.352
Protein carbonyl nmol/mg of protein	2.005	2.576	2.245	2.486	0.262	0.691	0.042	0.386
TNF-α, pg/mg of protein	1.313	1.600	1.285	1.149	0.229	0.263	0.718	0.321
IL-8, ng/mg of protein	1.569	1.706	1.590	1.285	0.197	0.324	0.674	0.276
IgA, pg/mg of protein	14.937	21.455	14.593	11.245	3.810	0.182	0.682	0.211
IgG, pg/mg of protein	5.201	6.075	5.243	5.855	0.830	0.916	0.382	0.876
Villus width, µm	196	186	199	206	9	0.201	0.854	0.343
Villus height (V), µm	338	343	402	351	31	0.269	0.480	0.384
Crypt depth (C), µm	316	297	299	311	17	0.923	0.821	0.353
V:C	1.08	1.17	1.33	1.14	0.10	0.276	0.607	0.161
Ki-67, %	28.1	29.2	29.7	27.0	2.0	0.878	0.682	0.352
Jejunum								
MDA, µM/mg of protein	1.451	1.208	0.742	0.645	0.183	<.0001	0.172	0.554
Protein carbonyl nmol/mg of protein	1.068	1.394	0.733	0.977	0.163	0.025	0.080	0.794
TNF-α, pg/mg of protein	0.453^{b}	0.872^{a}	0.699^{a}	0.744^{a}	0.078	0.460	0.008	0.027
IL-8, ng/mg of protein	1.372	1.219	0.831	0.920	0.126	0.003	0.803	0.347
IgA, pg/mg of protein	8.455	10.799	5.718	6.716	1.670	0.055	0.330	0.692
IgG, pg/mg of protein	3.168	4.126	3.516	3.681	0.414	0.908	0.191	0.350
Villus width, µm	155	162	155	158	9	0.821	0.543	0.840
Villus height (V), µm	354	349	342	308	23	0.246	0.387	0.528
Crypt depth (C), µm	254	280	234	240	15	0.053	0.273	0.501
V:C	1.44	1.26	1.48	1.29	0.14	0.754	0.128	0.963
Ki-67, %	38.2	39.6	35.7	39.9	3.3	0.745	0.401	0.683

MDA, malondialdehydes; TNF-α, tumor necrosis factor-alpha; IL-8, interleukin 8; IgA, immunoglobulin A; IgG, immunoglobulin G. Ki-67 is an estimate of the proliferative rate, calculated based on the proportion of nuclei positive to Ki-67 staining to the total nucleus number. Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). [a, b] Means within a row with a different superscript differ ($p < 0.05$).

The proximal jejunum microbiome sequencing on d 0 showed that light pigs had increased ($p < 0.05$) α-diversity at the family level according to Chao1, Shannon, and Simpson indexes (Table 9). At the genus level, light pigs had increased ($p < 0.05$) α-diversity according to Shannon and Simpson indexes, and tended to have higher ($p = 0.071$) α-diversity for the Chao1 index. At the species level, light pigs had increased ($p < 0.05$) α-diversity according to Shannon and Simpson indexes but no difference was observed for the Chao1 index. The microbiome profile showed that light pigs had decreased ($p < 0.05$) relative family abundance of Helicobacteriaceae (Table 10). On the other hand, light pigs tended to have a higher relative family abundance of Enterobacteriaceae ($p = 0.063$), Lachnospiraceae ($p = 0.067$), and the sum of families which individual relative abundances were less than 1% ($p = 0.094$), reported as "Other families" in the current study. There was no difference in the families Campylobacteraceae, Lactobacillaceae, Moraxellaceae, Prevotellaceae, and Veillonellaceae among pigs. Light pigs had lower ($p < 0.05$) relative genus abundance for *Helicobacter* but higher ($p < 0.05$) relative abundance for the sum of genera which individual relative abundances were less than 1%, reported as "Other genera" in the current study. There was no difference in the relative genus abundance for *Acinetobacter*, *Campylobacter*, *Lactobacillus*, *Megasphaera*, and *Prevotella* among pigs. Light pigs had lower ($p < 0.05$) relative species abundance for *Helicobacter mastomyrinus* and *Helicobacter rappini* but higher ($p < 0.05$) for *Prevotella stercorea*. Light pigs had higher ($p < 0.05$) relative abundance for the sum of species which individual relative abundances were less than 1%, reported as "Other species" in the current study. There was no difference in the relative species abundance for *Acinetobacter radioresistens*, *Brachyspira hampsonii*, *Campylobacter hyointestinalis*, *Helicobacter* sp.,

Lactobacillus kitasatonis, *Lactobacillus mucosae*, *Pelomonas puraquae*, *Prevotella copri*, and *Prevotella* sp. among pigs.

Table 9. Microbiome alpha-diversity in proximal jejunum of weanling pigs with different weaning weights on d 0.

Body weight	**< 7.5 kg**	**> 9.0 kg**	**SEM**	***p* Value**
Family				
Chao1	41.44	19.44	8.58	0.018
Shannon	3.35	1.42	0.33	0.004
Simpson	0.80	0.42	0.08	0.013
Genus				
Chao1	37.24	20.72	6.29	0.071
Shannon	3.34	1.32	0.33	0.004
Simpson	0.81	0.41	0.10	0.008
Species				
Chao1	44.76	25.27	8.51	0.149
Shannon	4.08	2.04	0.30	<0.001
Simpson	0.90	0.63	0.06	0.003

Table 10. Microbiome profile in proximal jejunum of weanling pigs with different weaning weights on d 0.

Body weight	**< 7.5 kg**	**> 9.0 kg**	**SEM**	***p* Value**
Family				
Campylobacteraceae	2.71	0.03	1.36	0.206
Helicobacteraceae	0.35	57.91	14.36	0.006
Enterobacteriaceae	22.09	1.53	9.78	0.063
Lachnospiraceae	5.15	0.44	1.54	0.067
Lactobacillaceae	15.94	13.46	8.83	0.848
Moraxellaceae	7.67	2.35	2.66	0.200
Prevotellaceae	11.41	9.84	4.86	0.827
Veillonellaceae	0.73	0.55	0.42	0.771
Other families	33.63	13.56	7.31	0.094
Genus				
Acinetobacter	11.19	1.14	4.23	0.136
Campylobacter	5.59	0.03	3.02	0.234
Helicobacter	1.39	59.68	15.09	0.006
Lactobacillus	23.64	15	10.52	0.58
Megasphaera	0.17	0.03	0.1	0.106
Prevotella	10.42	9.8	5.29	0.922
Other genera	46.31	13.04	7.79	0.019
Species				
Acinetobacter radioresistens	7.1	0.35	3.05	0.162
Brachyspira hampsonii	9.3	0.07	4.67	0.205
Campylobacter hyointestinalis	2.13	0.03	1.18	0.247
Helicobacter mastomyrinus	0.55	37.14	14.6	0.015
Helicobacter rappini	0.55	19.59	4.86	0.028
Helicobacter sp.	0.21	5.1	3.21	0.317
Lactobacillus kitasatonis	10.12	6.9	4.76	0.646
Lactobacillus mucosae	1.12	0.07	0.79	0.378
Pelomonas puraquae	1.71	5.98	4.01	0.469
Prevotella copri	11.21	12.61	7.03	0.842
Prevotella sp.	0.04	0.16	0.12	0.481
Prevotella stercorea	3.24	0.11	0.77	0.022
Other species	52.06	11.26	6.06	<0.001

All families, genera, and species that did not represent 1% of average bacterial diversity among all samples were grouped under the "Other families", “Other genera”, and “Other species” category, respectively.

At 20 kg of body weight, there was no difference in the α-diversity indexes assessed for family, genus, and species level among pigs (Table 11). The microbiome profile showed an interaction ($p < 0.05$) for the relative family abundance of Other families, where among heavy pigs, feeding high mycotoxins reduced ($p < 0.05$) the abundance of Other families, whereas there was no difference among light pigs (Table 12). There was no difference in the relative family abundance for Helicobacteraceae, Moraxellaceae, Prevotellaceae, Enterobacteriaceae, Veillonellaceae, Lactobacillaceae, Lachnospiraceae, and Campylobacteraceae among pigs. There was no difference in the relative genus abundance for *Lactobacillus*, *Mycoplasma*, *Acinetobacter*, *Helicobacter*, *Megasphaera*, *Campylobacter*, *Prevotella*, and Other genera among pigs. There was a tendency for interaction ($p = 0.066$) where among light pigs, feeding high mycotoxins increased ($p < 0.05$) the relative abundance for *Prevotella copri*, whereas there was no difference among heavy pigs. There was no difference in the relative species abundance for *Helicobacter rappini*, *Prevotella* sp., *Helicobacter* sp., *Campylobacter hyointestinalis*, *Brachyspira hampsonii*, *Lactobacillus mucosae*, *Pelomonas puraquae*, *Helicobacter mastomyrinus*, *Lactobacillus kitasatonis*, *Mycoplasma sualvi*, *Prevotella stercorea*, and Other species among pigs.

Table 11. Microbiome alpha-diversity in proximal jejunum of weanling pigs with different weaning weights and consuming diets with or without mycotoxins at 20 kg.

Body weight (BW)	**< 7.5 kg**		**> 9.0 kg**		**SEM**	***p* Value**		
Mycotoxins (MTX)	**-**	**+**	**-**	**+**		**BW**	**MTX**	**BW x MTX**
Family								
Chao1	20.96	23.53	27.57	21.32	5.47	0.690	0.739	0.428
Shannon	1.54	1.83	2.10	1.44	0.31	0.787	0.549	0.147
Simpson	0.46	0.53	0.62	0.45	0.09	0.669	0.586	0.181
Genus								
Chao1	19.16	23.55	23.24	21.14	5.86	0.851	0.797	0.468
Shannon	1.53	1.62	1.85	1.16	0.29	0.821	0.307	0.188
Simpson	0.46	0.49	0.58	0.38	0.09	0.993	0.374	0.206
Species								
Chao1	24.68	27.52	33.64	25.68	7.77	0.605	0.709	0.434
Shannon	2.32	1.94	2.34	2.12	0.29	0.707	0.254	0.767
Simpson	0.66	0.56	0.68	0.64	0.06	0.411	0.243	0.558

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7.5 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups).

Table 12. Microbiome profile in proximal jejunum in weanling pigs with different weaning weights and consuming diets with or without mycotoxins at 20 kg.

Body weight (BW)	**< 7.5 kg**		**> 9.0 kg**		**SEM**	***p* Value**		
Mycotoxins (MTX)	**-**	**+**	**-**	**+**		**BW**	**MTX**	**BW x MTX**
Family								
Helicobacteraceae	41.55	36.38	34.26	66.18	13.54	0.416	0.336	0.187
Moraxellaceae	0.00	0.17	0.33	0.36	0.26	0.323	0.706	0.774
Prevotellaceae	23.04	38.57	30.84	18.53	9.92	0.544	0.873	0.177
Enterobacteriaceae	13.60	0.02	0.31	1.38	6.02	0.328	0.305	0.232
Veillonellaceae	5.50	4.55	8.21	4.70	1.96	0.473	0.269	0.520
Lactobacillaceae	4.73	3.42	7.40	4.21	2.29	0.459	0.338	0.686
Lachnospiraceae	0.74	4.67	1.82	0.60	1.66	0.353	0.397	0.118
Campylobacteraceae	6.12	5.08	5.57	0.21	3.76	0.481	0.406	0.572
Other families	4.73[c]	7.14[bc]	11.26[ab]	3.82[c]	2.55	0.498	0.295	0.048
Genus								
Lactobacillus	7.29	4.29	9.12	4.57	3.09	0.736	0.236	0.805
Mycoplasma	1.03	0.00	2.89	1.93	1.73	0.249	0.538	0.982
Acinetobacter	0.00	0.13	0.33	0.33	0.24	0.284	0.773	0.796
Helicobacter	44.79	40.82	39.31	71.76	13.27	0.350	0.297	0.186
Megasphaera	1.58	0.95	2.89	0.85	0.88	0.498	0.142	0.429
Campylobacter	13.01	6.59	6.09	0.24	6.11	0.291	0.328	0.963
Prevotella	23.65	39.89	30.48	18.18	10.23	0.476	0.849	0.179
Other genera	8.65	7.33	8.90	2.15	2.72	0.376	0.155	0.331
Species								
Helicobacter rappini	12.28	6.60	2.23	10.79	4.65	0.536	0.760	0.142
Prevotella sp.	8.43	1.17	3.98	2.02	4.16	0.670	0.281	0.531
Helicobacter sp.	7.32	0.03	0.01	0.66	3.19	0.308	0.310	0.228
Campylobacter hyointestinalis	14.65	7.55	6.71	0.12	6.73	0.267	0.322	0.970
Prevotella copri	11.60[b]	42.79[a]	27.27[ab]	22.79[ab]	9.14	0.816	0.160	0.066
Brachyspira hampsonii	0.01	0.00	0.12	0.02	0.04	0.145	0.232	0.320
Lactobacillus mucosae	5.01	0.98	5.19	3.88	3.12	0.626	0.403	0.668
Pelomonas puraquae	0.88	0.21	1.60	0.14	0.75	0.674	0.174	0.607
Helicobacter mastomyrinus	0.18	0.27	0.28	0.40	0.12	0.318	0.401	0.875
Lactobacillus kitasatonis	0.01	0.01	0.02	0.02	0.01	0.292	0.880	0.959
Mycoplasma sualvi	0.01	0.00	0.04	0.03	0.02	0.236	0.533	0.945

Table 12. (Continued).

Body weight (BW)	< 7.5 kg		> 9.0 kg		SEM	*p* Value		
Mycotoxins (MTX)	-	+	-	+		BW	MTX	BW x MTX
Prevotella stercorea	0.03	0.03	0.02	0.03	0.02	0.894	0.754	0.737
Other species	0.16	0.10	0.16	0.11	0.05	0.889	0.291	0.906

Dietary treatments were based in 2 phases: phase 1 from d 0 until animals achieved 11 kg of body weight (4 d for pig weaning weight > 9 kg and 14 d for pig weaning weight < 7 kg) and phase 2 until pigs achieved 20 kg of body weight (17 d for both weaning weight groups). All families, genera, and species that did not represent 1% of average bacterial diversity among all samples were grouped under the "Other families", "Other genera", and "Other species" category, respectively. [a, b, c] Means within a row with a different superscript differ ($p < 0.05$).

5.5. Discussion

The variability observed in weaning weight on d 0 within the animals used in this study could be due to different weaning ages [4] or the use of artificial nursing methods during lactation [27]. However, all the pigs used in the current study were not provided supplemental nutritional source during the suckling period. Based on the weaning body weight of light pigs in the current study (average body weight of 6.9 kg) the weaning age is estimated at 21 days. For heavy pigs (average body weight of 9.8 kg) the weaning age is estimated at 35 days of age. Such values are in agreement with the weaning weight observed in a study conducted where the average weaning weight of pigs weaned at 21 and 35 days was 6.5 and 10.0 kg, respectively [4].

Because the pigs used in the current study had different initial body weights, performance data are shown by phase, to allow the comparison among factors and treatments. The difference in the initial body weight made light pigs spend 10 additional days to achieve the targeted body weight, 20 kg, by the end of the study. Phase 1 diet was fed from d 0 until pigs achieved 11 kg (4 days for heavy pigs and 14 days for light pigs), whereas for phase 2 both weaning weight groups took the same time, 17 days. The difference in body weight among weaning weight groups was

not expected by the end of phase 1. The targeted final body weight was set based on the time expected to elicit mycotoxin effects in the most recent and studies from our research team and nursery pigs with similar weaning weights, approximately 15 to 21 days [9,16].

The negative effect of feeding high mycotoxin diet (2 mg/kg of deoxynivalenol and 0.2 mg/kg of aflatoxins) on growth performance was observed by the end of the study with an average reduction of 2.1 kg in pig body weight. Light pigs had lower ADFI during phase 1, which could be explained by difficulties in adaptation to a solid and plant-based diet after weaning leading to lower ADG during phase 1. The effects of weaning age on ADFI and ADG of pigs is not new. It has been demonstrated that ADFI and ADG of nursery pigs are directly proportional to weaning age [2]. However, during phase 2, light pigs seemed to show a recovery in both ADFI and ADG. Such result could be related to the 10-day-longer phase 1 period in comparison to heavy pigs (14 vs. 4 days), thus, light pigs had more time to develop their gastrointestinal tract and were not challenged to the same extent by phase 2 diet formulation, with increased soybean meal percentage and reduced ingredients with high digestibility (milk-derived and animal-based ingredients). It was observed that pigs fed high mycotoxins had lower ADFI and ADG, but higher G/F, which could be an adaptation to better utilize the comparatively reduced amount of energy and nutrients ingested [28].

The higher fecal score observed on d 0, 10, and 20 in light pigs could be due to the difference in age among the two body weight groups. As demonstrated in a previous study [3], pigs with a lower weaning age showed an increased duration of diarrhea caused by weaning stress. Regarding the interaction observed for the fecal score on d 15, light pigs presented higher fecal scores following the same reason discussed before. But there was a mycotoxin effect to increase the fecal score of light pigs fed high mycotoxins in comparison to heavy pigs fed low

mycotoxins. An indecreased fecal score or increased diarrhea incidence is related to impaired intestinal health [29]. Thus, mycotoxins may have had impaired the health of the gastrointestinal tract of pigs and light pigs may be more susceptible to such mycotoxin toxic effects. Supporting the toxic effects of mycotoxins in the gastrointestinal tract, mycotoxins caused a decrease in dry matter and nitrogen apparent ileal digestibility in the current study. It was previously reported a decrease in dry matter and nitrogen apparent ileal in weanling pigs fed aflatoxins and deoxynivalenol [13]. Growing pigs also showed reduced apparent total tract digestibility of dry matter and nitrogen in feed under mycotoxin challenge [14]. The reduced gastrointestinal health and digestibility caused by mycotoxins could be caused by the impairment of cell metabolism. Deoxynivalenol has an inhibitory effect over protein synthesis at the cytoplasm through a mechanism known as ribotoxic stress [30] and may hinder SGLT1 expression and function [10,11]. On the other hand, aflatoxins inhibit messenger RNA synthesis impairing the activity of RNA polymerase at the nucleus [31]. However, feeding high mycotoxins increased ether extract digestibility among light pigs in the current study. It was previously shown that ether extract digestibility increases as time passes after weaning [2]. Thus, the increased ether extract digestibility could be partially due to the time elapsed after weaning as light pigs achieved 20 kg of body weight in 31 d after weaning, whereas heavy pigs took 21 d. Another factor could be the lower body weight in pigs fed high mycotoxins, as animals nutritionally restricted are more efficient in utilizing nutrients from feed [28].

The increased alkaline phosphatase in light pigs may suggest higher enterocyte differentiation to support growth. Alkaline phosphatase is indicative of enterocyte differentiation and functionality and its increase is linked to higher digestive and absorptive function [32,33]. Indeed, light pigs presented higher ADFI and ADG during phase 2. Light pigs had lower

chloride levels than heavy pigs. It is possible that light pigs were not absorbing chloride as effectively as heavy pigs or that there was an electrolyte leakage into the intestinal lumen. The poor absorption or electrolyte leakage could be caused by increased oxidative stress and local immune activation, which may have increased intestinal permeability [34]. However, no difference was observed for other electrolytes. Mycotoxins, as also seen in other variables measured, impaired protein synthesis decreasing albumin concentration and albumin to globulin ratio. Albumin is the main blood serum protein and, thus, can be used as an estimate of protein synthesis and hepatic function [35]. Interactions of weaning weight and mycotoxin factors showed that there was an increase in globulin in light pigs. Such an outcome is contradictory to the initial hypothesis and the outcomes observed for other variables (albumin, oxidative stress markers, nutrient digestibility, histometric measurements), showing the impairment over protein function caused by mycotoxins [6,7]. Creatinine was higher among heavy pigs fed mycotoxins, decreasing the blood urea nitrogen to creatinine ratio in these animals. Creatinine is indicative of hepatic function, where its increase could be related to hepatic damage and failure [15] caused by mycotoxins. Concerning the effects on electrolytes, the negative effect of aflatoxins on bone ossification because of its impact on vitamin D and calcium metabolism was previously shown [36]. The strict equilibrium maintained among calcium and phosphorus could have caused the reduction observed phosphorus concentration in heavy pigs fed mycotoxins. Nevertheless, no differences were observed among calcium serum levels.

On d 0, immune response and oxidative stress markers were greatly affected by weaning weight in the proximal jejunum rather than in the duodenum. In the duodenum, light pigs had increased malondialdehydes, indicating lipid peroxidation caused by oxidative stress [8]. Among immune response markers, light pigs had lower interleukin 8 but higher immunoglobulin G,

suggesting reduced cellular but increased humoral immune response [37,38]. Also, the villus width in light pigs was smaller in comparison to heavy pigs at weaning. Supporting the outcomes observed in the duodenum, the results from the proximal jejunum showed a similar trend but with more pronounced effects. The increased concentration of malondialdehydes and protein carbonyls in light pigs are both indicative of oxidative stress exceeding the antioxidative capacity of enterocytes [8]. The increased oxidative stress was likely caused by the increased inflammatory response, represented by the increased tumor necrosis factor-α [1]. In the proximal jejunum, a similar pattern was found with increased interleukin 8 and decreased immunoglobulin G in the duodenum, reinforcing a diminished local immune activation and, instead, a more pronounced systemic immune response [38]. Also, light pigs had a lower concentration of immunoglobulin A stressing the reduced recruitment of local immune cells [38]. Taking together duodenum and proximal jejunum measurements, light pigs seemed to be more susceptible to stress factors of weaning, showing increased inflammation, oxidative stress, and presenting higher local cellular immune response. The increased inflammation, oxidative stress, and systemic local immune response resulted in enterocyte damage and death. The loss of enterocytes can be inferred by the absence of significative difference in Ki-67 percentages among the body weight groups. Thus, suggesting that light pigs were not able to replace enterocytes damaged by weaning stress and resulted in decreased intestinal surface [1] (villus width and villus height) and proliferation (crypt depth and villus height to crypt depth ratio) in light pigs.

At 20 kg of body weight, like the outcomes observed on d 0, the immune response, oxidative stress markers, and histometric measurements were more influenced by weaning weight and mycotoxin factors in the proximal jejunum than in the duodenum. In the duodenum, a

lower concentration of malondialdehydes showed in light pigs was unexpected, especially when put aside to the higher oxidative stress markers (malondialdehydes and protein carbonyls) in the proximal jejunum of light pigs. However, it can be hypothesized that it was due to the longer duration of the nursery phase for light pigs, so light pigs had more time to acclimate to the detrimental effects weaning had on oxidative stress. Mycotoxins increased protein carbonyls in the duodenum suggesting increased oxidative stress [8]. The fact that only proteins were affected by oxidative stress could be due to the impaired RNA transcription and translation caused by aflatoxins and deoxynivalenol [30,31]. Therefore, other cell constituents may have had the ability to be repaired or replaced, whereas it did not happen to the same extent for proteins. In the proximal jejunum, the higher malondialdehydes and protein carbonyls in light pigs, suggests that light pigs were experiencing a greater and long-lasting challenge 31 d after weaning than heavy pigs 21 days after weaning. Differently to what was observed on d 0, light pigs seemed to have more pronounced local immune activation, as seen by the greater concentrations of interleukin 8 and immunoglobulin A [37,38]. Mycotoxins increased oxidative stress in the proximal jejunum, based on the increase in protein carbonyls [8]. Of note, light pigs may be more prone to inflammatory effects caused by mycotoxins as light pigs fed mycotoxins showed increased tumor necrosis factor-α, whereas no difference was observed among heavy pigs. The detrimental effects of weaning stress and mycotoxin challenge in light pigs caused an increment in crypt depth. Suggesting an increased cell proliferation to provide enterocytes to maintain villus height [1] and their digestive, absorptive, and barrier function. Also, the lack of difference in Ki-67 counting along with the significant difference in crypt depth observed in the current study highlight the need to combine the outcome of both measurements when assessing enterocyte

proliferation, as the first is a proportion of actively dividing cells in a given moment and the latter is an estimate of the number of cells with the potential to multiply.

On d 0, the increased α-diversity observed in light pigs for family, genus, and species level can be related to the fact that the microbiome community was not established in this group of animals in comparison to heavy pigs. The increased α-diversity could be noticed at the family, genus, and species levels, where light pigs showed an increased relative abundance of Other families, Other genera, and Other species, indicating a larger proportion of the microbiome is composed of diversified bacteria but with few individual microbes within the same organizational levels. An increased microbial diversity was recently reported in rectal swabs of pigs with 20 days of age, prior to weaning [5], which is close to the estimated age based on the body weight for the light group observed in the current study. The same authors also demonstrated that a decrease in α-diversity was observed right after weaning (21 days of age) followed by a steady and slow increase until pigs reached market weight. However, the weaned pigs were fed diets containing zinc in the referred study, whereas there was no inclusion of zinc in the nursery diets in the current study because of its effect reducing microbiome diversity in the post-weaning period [39]. Also, an increased relative abundance was observed for *Prevotella stercorea* in light pigs. Such a result is in agreement with the increased relative abundance from 11 to 20 days of age for *P. stercorea* in suckling piglets [5]. The decreased relative abundance of the Helicobacteriaceae family for light pigs was followed by decreased relative abundances for *Helicobacter* genus as well as for the species of *H. mastomyrinus* and *H. rappini*. This outcome was unexpected, as bacteria from the *Helicobacter* genus are commonly recognized as opportunistic pathogens [40]. Also, light pigs showed higher proportions of their microbiome from the families Enterobacteriaceae and Lachnospiraceae. Similar outcomes were reported by

Frese et al. [41], where Enterobacteriaceae and Lachnospiraceae families were the ones with higher abundance in suckling piglets. However, the same study demonstrated that the Enterobacteriaceae family had its relative abundance decreased after weaning and as animals matured, whereas the Lachnospiraceae family remained relatively constant. In nursery pigs, an increased relative abundance in the Lachnospiraceae family was related to increased ADFI [42]. Even though milk consumption by pigs was not estimated in the current study, it can be inferred that light pigs were likely to be consuming a reduced amount of milk in comparison to heavy pigs. Thus, in the current study, the positive relationship between feed intake and Lachnospiraceae family abundance was not sustained in the case of suckling piglets.

The influence of feed change, from sow milk to solid feed, on pig microbiome was reported to start as early as 8 days after weaning [5]. Hence, the dietary treatments provided for 21 or 31 days for light or heavy pigs, respectively, were expected to influence the microbiome profile assessed in the current study. The increased relative abundance for *P. stercorea* observed in light pigs on d 0 disappeared at 20 kg of body weight, a similar reduction was observed in a longitudinal study when comparing pigs sampled before weaning and during the nursery period [5]. Regarding the increase in the relative abundance for *P. copri* during the nursery phase in light pigs fed mycotoxins, a similar outcome was described by Wang et al. [12] with an increase in *Prevotella* genus in the cecum and colon of weaned pigs fed deoxynivalenol for 28 days (versus 31 days for light pigs in the current study). Also, an increased abundance of *P. copri* was observed right after the introduction of solid feed [5]. However, there seems to be a decrease for the *Prevotella* genus [43] with age, suggesting that the increase can be related to the change to a solid and plant-based diet and the following decrease could be related to the establishment of the gut microbial community. Comparing suckling piglets and nursery pigs, Frese et al. [41] reported

an increased relative abundance of Prevotelaceae family in nursery pigs. Suggesting that changes in Prevotelaceae family abundance can be related to the diet [5], instead of the animal age during the suckling period as piglets showed no difference until 28 days of age [41]. In the current study, there were no differences in the family or genus levels. The increased relative abundance for *P. copri* among light pigs fed high mycotoxins at 20 kg of body weight may indicate that light pigs fed high mycotoxins took an additional time to establish their microbial community in comparison to heavy pigs and to light pigs fed low mycotoxin diet. Similar to the findings in the current study, *P. stercorea* has been found to have increased abundance in suckling pigs, whereas *P. copri* has been found to have increased abundance during the nursery phase even though both species belong to the *Prevotella* genus [5]. However, the lack of difference in the α-diversity indexes at 20 kg of body weight may indicate that both light and heavy pigs have achieved similar maturation levels in their respective microbial communities in the gut and, therefore, they did not differ at this time point. Likewise, challenging nursery pigs with deoxynivalenol (8 mg/kg of feed) did not result in differences in microbial diversity in the gut using the same indexes tested in the current study (Chao1 and Shannon) and the phylogenetic diversity whole tree [44]. Taking the higher proportion of *P. copri* in light pigs fed high mycotoxins and the absence of difference in the α-diversity indexes may suggest that *P. copri* can serve as a single, and perhaps, more precise indicator than the overall microbiome diversity regarding microbiome community establishment particularly in the case of mycotoxin challenge. Pig microbiome profile was expected to concentrate, reducing the variability as pigs aged [43]. However, unexpectedly, heavy pigs fed low mycotoxin diet had higher relative abundance for Other families than heavy pigs fed high mycotoxins and light pigs fed diets without additional mycotoxins.

5.6. Conclusion

Weaning light pigs (weaning weight < 7.5 kg) impaired health independent of dietary mycotoxin (supplemental aflatoxins at 0.2 mg/kg and deoxynivalenol 2.0 mg/kg of feed) challenge, whereas mycotoxins impaired nutrient digestibility, health, and growth performance independent of weaning weight. Light pigs had immature microbiome upon weaning but became similar to heavy pigs (weaning weight > 9.0 kg) when both achieved 20 kg. Of interest, the proximal jejunum of weaned pigs seems to be more susceptible to weaning stress and mycotoxin challenge than the duodenum.

5.7. References

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CHAPTER 6. GENERAL CONCLUSIONS

The swine industry is under constant remodeling to improve efficiency for producers and to attend to customer's needs. Mycotoxins are substances produced by fungal metabolism with potential detrimental effects when fed to pigs. Aflatoxins, deoxynivalenol, and fumonisins are the mycotoxins with major impact in the swine industry because of their frequency, their toxic effects, and the existence of official regulations. The impact of mycotoxins in the swine industry, the growing mycotoxin contamination levels, and the need to optimize the utilization of food and feed products are reasons which turn the investigation and use of feed additives to mitigate the toxic effects of mycotoxins advantageous. At the same time, customers are demanding healthy, sustainable, and natural pork meat. The use of yeast additives in the swine industry to promote health and growth emerged from giving a second use to yeast by-products from the breweries and baking industry. Therefore, the use of yeast additives to mitigate mycotoxin toxic effects combine the needs of the swine industry with the requirements to satisfy customer claims.

Despite promising results of yeast additives in improving the intestinal architecture, enhancing immune function, and favoring the growth of beneficial bacteria, there are still inconsistencies in its use as an additive to mitigate mycotoxin toxic effects. Species, strain, processing, and concentration of the yeast additive are factors that may influence the outcomes. Also, the type(s) of mycotoxin(s), the mycotoxin concentration, pig age, as well as environmental conditions can affect the results observed in research trials testing yeast-based additives to mitigate mycotoxin toxic effects. Thus, this research focused on investigating the effects of different yeast-based additives to mitigate mycotoxin toxic effects in weanling pigs fed diets naturally contaminated with major mycotoxins. Also, mycotoxin toxic effects were investigated in weanling pigs with different weaning weights.

In the first study the effects of mycotoxins, the effects of a yeast-based additive (postbiotic yeast cell wall-based blend, PYCW) alone, and the effects of the PYCW to mitigate mycotoxin toxic effects were investigated. Particularly in this first study, aflatoxin-contaminated corn was added only to the phase 3 diet to model a real scenario in a commercial farm, where pigs may be fed diets with higher mycotoxin levels as they get older. Mycotoxins negatively impacted ADFI, ADG, and G:F of pigs. The reduced ADFI caused by mycotoxins is caused by a combination of hormonal and cytokine signaling which all lead to a satiety feeling. As evidenced, the reduction in ADG observed was greater than the reduction in ADFI, suggesting that pigs have reduced gain not solely because of nutritional deprivation. Indeed, it was observed reduced apparent ileal digestibility of nutrients in feed. The PYCW used to mitigate mycotoxin toxic effects did not show a clear improvement on growth performance, but the additive hindered the reduction in ADG and enhanced G:F in pigs challenged with mycotoxins during the first week. However, the PYCW reduced nutrient digestibility and ended in reducing G:F. On organ health, mycotoxins impaired hepatic protein and cholesterol synthesis as well as affected hepatic enzymes which were mitigated by the PYCW. Even though mycotoxins and yeast derivatives may impact oxidative stress and immune function of pigs, no difference was observed in the variables assessed. On intestinal architecture, the decrease in crypt depth was the only noticeable effect caused by mycotoxins and which could be related to the inhibitory effect over cell metabolism and proliferation.

In the second study, pigs were challenged only with deoxynivalenol to eliminate the confounding effect of multiple mycotoxin challenge. Also, diverse yeast-based additives to mitigate mycotoxin toxic effects were tested. The choice of deoxynivalenol was based on its major impacts in the swine industry in addition to the lack of an efficient feed additive to

mitigate the deleterious effects caused by deoxynivalenol. Three products were tested (CYC, CYE, and CYB) having in common variable sources of yeast. Deoxynivalenol caused oxidative stress in jejunal mucosa and its negative effects on growth performance could be noticed after 21 d of feeding mycotoxin contaminated diet. Pigs challenged with deoxynivalenol and fed CYE (clay/yeast cell wall/plant extracts/antioxidants-based product) showed a higher fecal score and blood urea nitrogen to creatinine ratio than pigs challenged with the mycotoxin. Pigs challenged with deoxynivalenol and fed CYC (clay/yeast culture-based product) showed improved G:F and ADG suggesting improved gut health. Challenged pigs fed CYC and CYB (clay/inactivated yeast/botanicals/antioxidants-based product) showed decreased immune activation in comparison to challenged pigs, which may indicate the role of yeast metabolic products or intracellular components in modulating immune function in jejunal mucosa. Challenged pigs fed CYB showed lower oxidative stress in comparison to challenged pigs. However, challenged pigs fed the three additives had lower serum albumin. Overall, the yeast-based additives with the whole-cell in combination with mineral adsorbents, plant-derived compounds, and antioxidants seem to have more beneficial outcomes than using yeast cell wall extract in deoxynivalenol challenged pigs.

The third study investigated another yeast-based product in weanling pigs challenged with deoxynivalenol but, differently to other products tested, this one included an enzyme complex. Similar to the second, the third study could not demonstrate a consistent impairment in pig growth performance. Besides the reduction in averaged daily gain during week 5 in pigs fed deoxynivalenol, there was no further deoxynivalenol effect observed on growth performance. Challenged pigs fed the yeast and enzyme-based additive had a reduction in feed intake during week 1 and increased averaged daily gain during week 4 than challenged pigs. The improvement

observed in growth performance in pigs challenged with deoxynivalenol could be due to the multiple component-approach (activated aluminosilicate, autolyzed yeast, probiotic yeast culture, calcarium marine algae powder, and enzyme complex) in the additive. Deoxynivalenol slightly impaired liver health and reduced anti-oxidant compounds in jejunal mucosa, which was recovered by the yeast and enzyme-based additive.

Considering that our research group has been performing many mycotoxin studies, the number of replications and deoxynivalenol concentration were expected to result in impaired pig growth in the second and third studies. Therefore, it was hypothesized that deoxynivalenol concentration in the diet of non-challenged pigs (1 mg/kg of feed), could have led to some impairment on pig growth performance and masked the outcomes in challenged pigs. A second hypothesis was formulated based on the first study (where pigs challenged with mycotoxins showed strong impairment on growth performance after 1 week when the average initial body weight was 7.5 kg) and the second and third studies (where pigs challenged with deoxynivalenol took 21 days or did not show growth impairment at all when the average initial body weight was 8.2 kg). Thus, the fourth study was designed to test if the difference in weaning weight (7.5 kg in the first study and about 8.2 kg in the second and third studies) would influence the susceptibility of weanling pigs to mycotoxins. The outcomes showed that light pigs (initial body weight < 7.5 vs. > 9.0 kg) were more susceptible to inflammation and had impaired intestinal health due to weaning stress, whereas mycotoxins impaired the health and growth of pigs regardless of weaning weight.