RICARDO MIRANDA GARCIA

APPROACHES IN GUT HEALTH AND ADDITIVES FOR YOUNG PIGS

LAVRAS - MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para obtenção do título de Doutor.

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RICARDO MIRANDA GARCIA

APPROACHES IN GUT HEALTH AND ADDITIVES FOR YOUNG PIGS

ABORDAGENS EM SAÚDE INTESTINAL E ADITIVOS PARA LEITÕES JOVENS

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Não Ruminantes, para obtenção do título de Doutor.

Aprovada em 29 de março, 2021.

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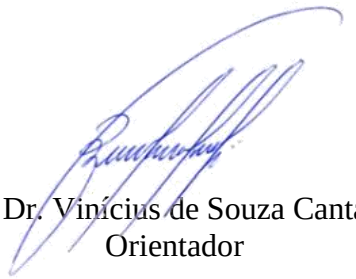

Prof. Dr. Vinícius de Souza Cantarelli
Orientador

LAVRAS - MG
2021

Aos meus pais Frassinetti e José, pela inspiração.

À minha esposa Amanda, pelo apoio em todos os momentos.

À minha filha Ritinha, minha felicidade.

Dedico.

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RESUMO

Três experimentos foram conduzidos para avaliar os efeitos de aditivos via ração e água sobre o desempenho e saúde intestinal de leitões em fase de creche. O objetivo do experimento 1 foi avaliar os efeitos de ácidos orgânicos (AO) via água. Cento e doze leitões (5,17 kg, aproximadamente 20 d de idade) foram blocados e divididos em quatro tratamentos e sete repetições: controle negativo (CN, dieta basal); controle positivo (CP, NC + 150/120/80/80 mg/kg de colistina nas quatro fases, respectivamente); e duas dosagens de AO/litro de água, AO1 (1,0 mL AO/L), e AO2 (2,0 mL AO/L). A inclusão de AO na água aumentou o consumo de água, a concentração de ácidos graxos voláteis no conteúdo cecal e a capacidade antioxidante no jejuno, mas não afetou o desempenho e a incidência de diarreia. O objetivo do experimento 2 foi investigar os efeitos de *dacitic tuff breccia* (DTB) na dieta de leitões na fase de creche (7,73 ±0,21 kg; 28 ±0,5 d). Os tratamentos foram: 1) dieta controle (CON); 2) CON + 0,25% DTB (DTB1); e 3) CON + 0,50% DTB (DTB2). Amostras de sangue, jejuno e íleo foram coletadas para análises metabólicas e histológicas. Leitões do grupo DTB2 tiveram uma tendência de maior ganho de peso diário (P=0,07) comparado a aos grupos CON e DTB1, e maior consumo de ração diário (P=0,008). A eficiência alimentar não diferiu nos tratamentos (P=0,75). A profundidade de cripta no jejuno foi maior para DTB1 comparado ao grupo CON mas não diferiu do DTB2 (P=0.04). A histomorfometria, número de células de Goblet e linfócitos intraepiteliais não diferiram entre os tratamentos (P>0,17). A proporção de monócitos foi menor em leitões do grupo DTB1 comparado ao CON e DTB2 (P=0,024). A concentração de ureia no sangue diminuiu linearmente com o aumento da concentração de DTB na dieta (P=0,06). O objetivo do experimento 3 foi avaliar a digestibilidade de nutrientes de dietas contendo 0,5% DTB. Os tratamentos foram: 1) Controle (CON) e 2) CON+0,50% DTB (DTB). Os leitões (7,73±0,21 kg; 26±0,2 d) foram individualmente alojados e alimentados por 20 dias. A digestibilidade de nutrientes foi avaliada utilizando gaiolas de metabolismo para coleta total de fezes e urina por três dias. No período total (d 0-20), o grupo DTB teve maior ganho de peso diário (P=0,04) e peso final (P=0,04) quando comparado ao CON. A digestibilidade de nutrientes não foi afetada pela inclusão de DTB na dieta (P>0,05), exceto pela melhor digestibilidade de nitrogênio nos leitões do grupo DTB (P>0,05). Em conclusão, DTB na dieta de creche pode maximizar o desempenho de leitões e AO pode ser utilizado como alternativa ao uso de colistina, não afetando o desempenho, mas melhorando a capacidade antioxidante no jejuno.

Palavras-chave: enzimas antioxidantes; diarreia; *dacitic tuff breccia*; digestibilidade.

ABSTRACT

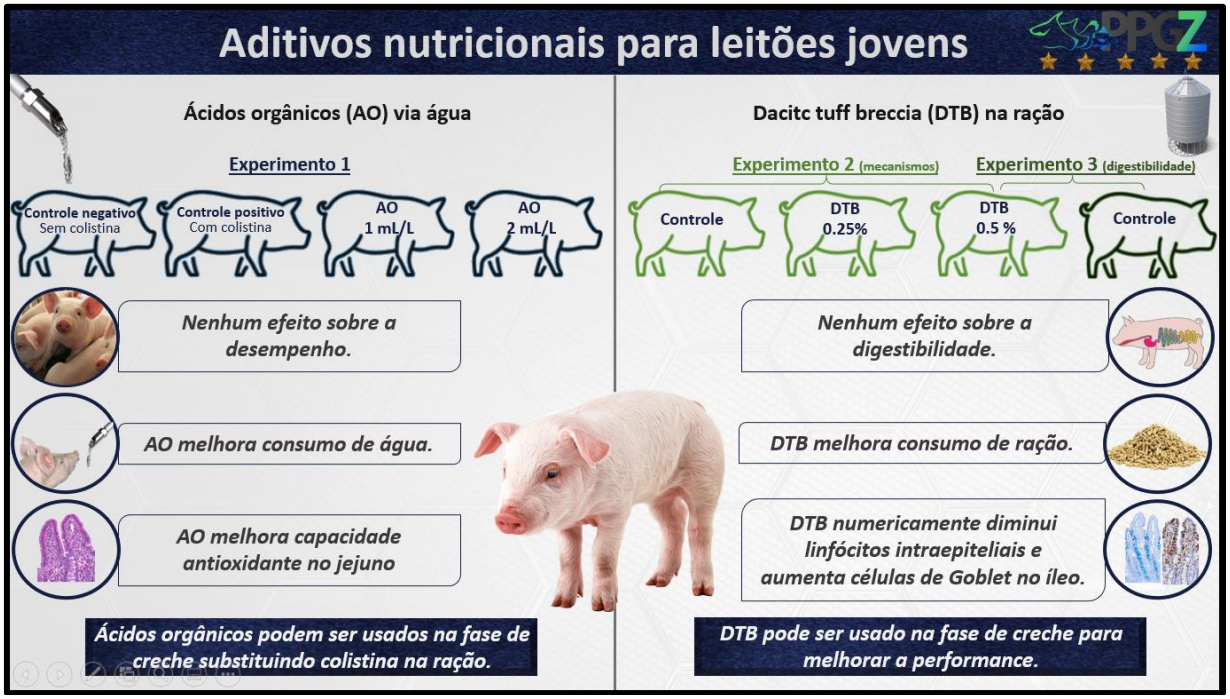
Three experiments were conducted to evaluate the effects of feed and water additives on nursery pig growth performance and intestinal health. The objective of experiment 1 study was to investigate the effects of organic acids (OA) in the drinking water. One hundred-twelve weaned pigs (5.17 kg, approximately 20 d old) were assigned in a completely randomized block design with four treatments and seven replicates: negative control (NC, basal diet), positive control (PC, NC + 150/120/80/80 mg/kg of colistin in each of four phases, respectively), and two doses/liter of water of OA, OA1 (1.0 mL OA/L), and OA2 (2.0 mL OA/L). The inclusion of OA in the water increased daily water usage, volatile fatty acids concentration in cecum content, and the overall antioxidant capacity of jejunum but did not affect growth performance and diarrhea incidence. The objective of experiment 2 was to investigate the effect of a dacitic tuff breccia (DTB) in the diet of nursery pigs (7.73±0.21 kg; 28±0.5 d). Dietary treatments were: 1) Control diet (CON), 2) CON + 0.25% DTB (DTB1), and 3) CON + 0.50% DTB (DTB2). Blood, jejunal, and ileal samples were collected for a typical metabolic panel and basic histologic and morphologic measurements. Pigs fed DTB2 tended to have greater average daily gain (P=0.07) than CON and DTB1 fed pigs, and greater average daily feed intake (P=0.008). Gain:Feed did not differ among treatments (P=0.75). Crypt depth in the jejunum was greater (P=0.04) for DTB1 compared to CON fed pigs but did not differ from DTB2 fed pigs. Histomorphometry, number of goblet cells and intraepithelial lymphocytes were not different among treatments (P>0.17). The proportion of monocytes was lower in DTB1 compared to CON and DTB2 fed pigs (P=0.024). Blood urea nitrogen concentration tended to be linearly decreased with increasing DTB concentrations (P=0.06). The objective of experiment 3 was to evaluate nutrient digestibility of diets containing 0.0% or 0.5% DTB. Dietary treatments were: 1) Control (CON) and 2) CON+0.50% DTB (DTB). Pigs (7.73±0.21 kg; 26±0.2 d) were individually housed and fed for 20 d (n=24). Nutrient digestibility was evaluated using individual metabolism pens for 3 d total collection of feces and urine during last week of the study. Overall nutrient digestibility was not affected by inclusion of DTB in the diet (P>0.05), accept better nitrogen retention for DTB fed pigs (P<0.05). In conclusion, DTB in nursery diets can maximize growth performance of nursery pigs and OA can be used in alternative to colistin not altering growth but improving antioxidant capacity in the jejunum.

Keywords: antioxidant enzymes; diarrhea; nutrition; dacitic tuff breccia; digestibility.

RESUMO INTERPRETATIVO

Estratégias para minimizar os impactos do desmame sobre a saúde e desempenho de leitões na fase de creche tem sido alvo de estudos. O período pós-desmame é considerado o mais desafiador no ciclo de vida de um suíno. Nesse momento, os animais enfrentam diversos fatores estressores como a separação da mãe, a mistura com outros animais, introdução de uma nova dieta e novo ambiente. Dois diferentes aditivos, um via água e outro via ração, foram testados em três experimentos. No experimento 1, ácidos orgânicos via água de bebida foram avaliados como alternativos ao uso de colistina na ração. Foi observado que ácidos orgânicos na água de bebida tiveram um efeito positivo na capacidade antioxidante no jejuno e afetaram a concentração de ácidos graxos voláteis no conteúdo cecal. Nenhum efeito sobre o desempenho foi observado nesse estudo. Ácidos orgânicos para leitões desmamados podem ser utilizados como uma estratégia para diminuir o pH da água de bebida e melhorar parâmetros de saúde intestinal. Nos experimentos 2 e 3, foi avaliada a inclusão de *dacitc tuff breccia* (DTB) nas dietas de creche. Nenhum efeito sobre parâmetros sanguíneos e metabólicos foi observado, garantindo a segurança do aditivo. Foi demonstrado que DTB melhorou o consumo de ração e o desempenho dos animais mesmo não afetando digestibilidade de nutrientes. Diferentes abordagens no uso de aditivos podem ser utilizadas para melhorar saúde e desempenho de leitões jovens.

RESUMO GRÁFICO

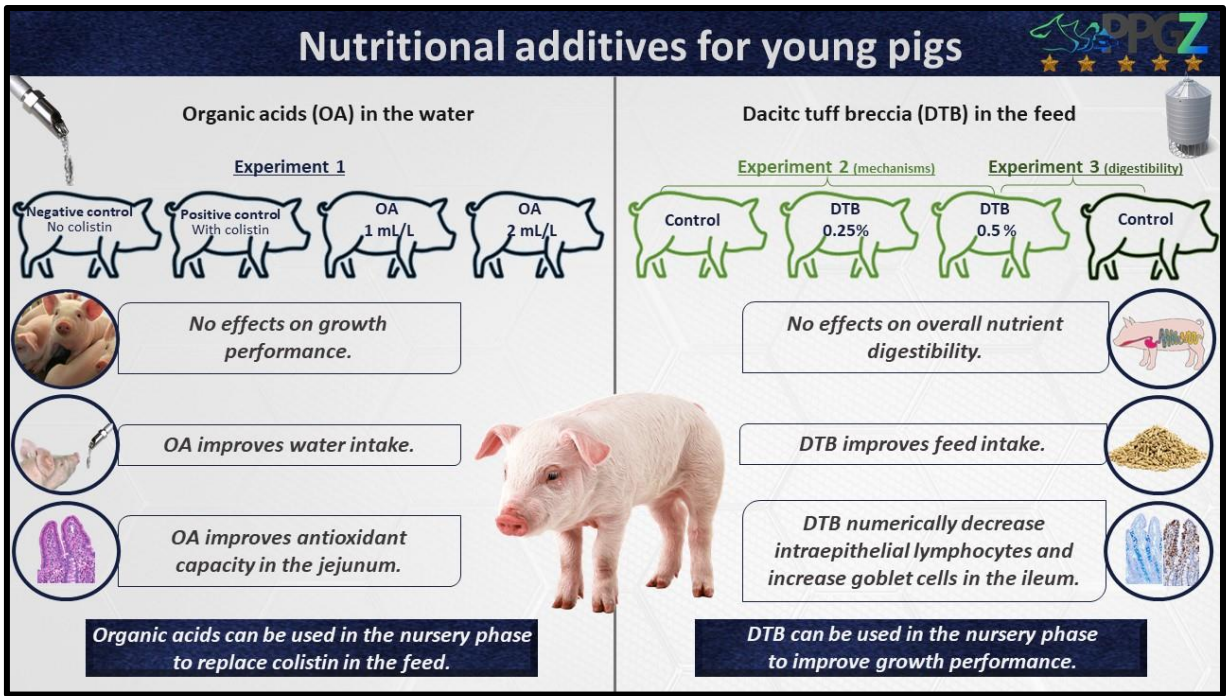


O período pós-desmame é considerado o mais desafiador no ciclo de vida de um suíno. Sendo assim, existe necessidade de estratégias que minimizem os impactos negativos do desmame sobre a saúde e desempenho de leitões em fase de creche. Três experimentos foram conduzidos para avaliar o uso de diferentes aditivos para leitões na creche. Foi demonstrado que ácidos orgânicos via água de bebida podem ser uma alternativa ao uso de colistina na dieta. Além disso, *dacitic tuff breccia* é um aditivo seguro para melhorar o desempenho de leitões jovens.

INTERPRETIVE SUMMARY

Many strategies have been studied to ameliorate the negatives impact on health and growth performance of weaning on nursery pigs. The post-weaning period is characterized as the most challenging phase of a pig's life cycle. At this moment, pigs are facing several stressors such as the separation from the sow, co-mingling with other animals, and the contact with a new diet and environment. Two different additives, one in the water and another in the feed, were tested in three experiments. In experiment 1, organic acids in the drinking water were evaluated as alternative to colistin in the feed. The results showed that organic acids had a positive effect on overall antioxidant capacity in the jejunum and affects volatile fatty acids concentration in cecum contents. No effects on growth performance were observed in this study. Organic acids in the water for weaned pigs can be used as a strategy to lower drinking water pH and improve intestinal health parameters. In experiments 2 and 3, the inclusion of dacitic tuff breccia (DTB) was evaluated in the nursery diets. No effect on blood and metabolic parameters was observed which ensure its safety application for pigs. It was demonstrated that DTB improved feed intake and the growth performance of pigs while not affecting overall nutrient digestibility. Different approaches on additives utilization can be used to improve health and growth performance of young pigs.

GRAPHICAL ABSTRACT



The post-weaning period is characterized as the most challenging phase of a pig life cycle. Therefore, there is need of strategies to mitigate the weaning negatives impacts on health and growth performance of nursery pigs. Three experiments were conducted to evaluate the use of different additives for nursery pigs. It was demonstrated that organic acids in the drinking water can be an alternative to the use of colistin in the diet. In addition, dacitic tuff breccia is a safe feed additive to improve growth performance of young pigs.

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SUMMARY

CHAPTER 1

1 INTRODUCTION

Pork is the most animal meat consumed worldwide and can be considered the main protein source for billions of people while generating millions of jobs across the industry. Animal products are conditioned to consumers demands which drives the global trends regarding welfare and food safety. The swine industry is constantly facing multiple threats in the different chain segmentations such as the suppliers, pig producers, the processing plants, and ultimately consumers.

Feed ingredients are the most important part of the costs of pig production. For instance, crystalline amino acids and vitamins are often collapsing in prices and in availability as well as geopolitical threats affecting prices and trade, which ultimately forces the industry to become even more efficient to ensure profitability. Nevertheless, to overcome other challenges that are intrinsic to the pork production, a lot of efforts from the industry and researchers have been leading to innovation on new ingredients, additives, genetics improvements, and management. Alternatives to antibiotics, hyper prolific sows and high pre-weaning and sow mortality rates, body weight variability, and overall pig survivability are current examples of topics that have been largely explored to improve profitability and welfare. The weaning process is also subject of several studies since it affects the performance and health of pigs.

Nutritional and management strategies that minimize weaning negative impacts on pig health, welfare, and performance are important to pork production. At weaning moment, pigs are vulnerable to stress consequences caused by nutritional, environmental, social, and immunological changes. The negative impacts on nursery piglet performance are related to intestinal injuries, increased gut permeability, microbiota imbalance, and decreased nutrient digestibility and absorption.

The gastrointestinal tract (GIT) barrier plays an important role in the overall pig health. Layers from different systems such as the nervous, epithelial, and immune systems constitute a barrier defense to the hostile environment produced by the luminal contents, which includes pathogens and antigenic compounds. That happens at the same time of controlling normal functions of the intestine like digestion and absorption of nutrients. Breakdown of GIT barrier function is a central cause for much of the enteric issues in pigs on first few weeks post-weaning, and those are a major reason for antibiotic use in piglet’s diets after weaning.

There is a need for nutritional strategies to support pig growth after weaning and during the nursery phase. Feed and water additives, functional ingredients, and pig management are applied to replace antibiotics utilization as growth promoters, which is one of the most important concerns on global livestock production. The aim of this literature review is to synthesize research regarding the utilization of dietary additives that promotes growth and health of pigs such as organic acids, trace minerals, and mycotoxicosis ameliorators.

2 LITERATURE REVIEW

2.1 Effects of weaning on intestinal health

The weaning process is considered and studied as the most challenging moment in the pig's life. It is the time to face nutritional, environmental, immunological, and social stressors factors that may impact their whole life. Together these stressors lead a breakdown of the GIT barrier and compromise the gut health.

Bischoff (2011) defined five major principles that could form the explanation of a definition for intestinal health: effective nutrients digestion and absorption, absence of GIT illness, microbiome equilibrium, effective immunological functions, and status of well-being. The weaning may disrupt all the criteria that define a healthy GIT, it modifies enzyme activity (MONTAGNE et al., 2007) and nutrient bioavailability (SUTHONGSA et al., 2017), impairs intestinal morphometry (MOESER et al., 2007; SUTHONGSA et al., 2017), promotes modification of microbiota population (ISAACSON and KIM, 2012; BAUER et al., 2006; POULSEN et al., 2018), super activates the immune system (PIÉ et al., 2004; PLUSKE et al., 2018), and modifies the normal behavior of suckling pigs (TURPIN et al., 2017).

After birth, the GIT of pigs undergoes a maturation processes including changes in size (LINDEMANN et al., 1986), barrier function (TAKEDA et al., 2004), enzymatic secretion (LE HUËROU-LURON et al., 2002), microbiota colonization (KUBASOVA et al., 2018), and immunological functions (BAILEY et al., 2005; WILSON et al., 2005). The GIT development is a plastic process, then may be modified by environmental stimulus. Thus, the weaning stressors factors might shape the GIT development and its functions are affected by the age that weaning occurs (MOESER et al., 2017). During this time, intestinal epithelial and enteric nervous system phenotype and function change dramatically as the pig adapts to the extra-uterine life at the same time of immunological window occurs. Whereas some developmental changes are a result of intrinsic genetic programming or biological clocks, many changes are influenced by changing environmental cues. Many developmental processes exhibit a high degree of plasticity during this time and thus challenges occurring in this critical window can largely shape the long-term phenotype and GIT function (MOESER et al., 2017).

Pigs are separated from the sow, transported, moved from a place to another, have the first contact with dry feed, mixed with other pigs creating social stress, and are exposed to

pathogens and dietary antigens. The pig must adapt to all the stressors mentioned above very quickly to be productive and efficient (CAMPBELL et al., 2013).

Feed intake is the major affected behavior, and it is the start for many other consequences in health and performance. At weaning, many factors will affect feed intake such as the age, having or no access to creep feed before, the health status, the environment, stock density, and those related to the diet like formulation, nutrients level and balance, palatability, forms, water supply and quality (DONG and PLUSKE, 2007). Brooks et al. (2003) reported that 50% of the weaned pigs eat for the first time within 24 hours and 10% eat only after 48 hours. The metabolizable energy intake varies around 60-70% of the energy intake from the sow milk in the pre-weaning period during the first week after weaning (LE DIVIDICH et al., 2000).

As a result of early weaning stressors and a low feed intake, the intestinal barrier functions changes in its morphology and permeability (POHL et al., 2017), including villous atrophy which reduces the capacity of nutrients digestion and absorption (McLAMB et al., 2013).

Marsi et al. (2015) found that the impacts on intestinal morphology are notable in weaned pigs aged less than 28 days and that the recovery of villous atrophy may take around 14 days. The villous:crypt ratio is significantly lower in weaned pigs than in suckling pigs (PLUSKE et al., 2003) and it is an important parameter to evaluate the intestinal mucosa quality, since higher values for this ratio indicate less mucosal injury. Gu et al. (2002) showed that five days after weaning, the villous damage was higher in 17 day old weaned pig, and 11 days were necessary to recover the villous height compared to other evaluated ages. Moreover, in animals weaned at 28 days there was no reduction in villous height.

The pre-weaning microbiota composition is modulated by many factors including maternal microbiota, GIT pH, substrate availability, peristalsis, mucus secretion, milk and creep feed intake (KIM et al., 2012), and after weaning, several factors may contribute for losses in microbiota diversity.

The dietary changes from milk with great fat and low carbohydrates to solid feed with great carbohydrate and low fat are known as important drivers in the modulation of gut microbiota in pigs in the post-weaning period. A well-balanced microbiota acts in carbohydrates fermentation, producing short-chain fatty acids (SCFAs) and vitamins, and plays an important role in maintaining normal functions in intestine, such as inhibition of pathogens proliferation and immune response modulation (ISAACSON and KIM, 2012).

Guevarra et al. (2018) reported a great increase in *Prevotella* after weaning which is related to plant-derived non-starch polysaccharides (NSPs) fermentation to SCFAs. The relative abundance of *Lactobacillus* was greater in weaned pigs as well, and that may play a role in the complex carbohydrate ‘fermentation allowing pigs to adapt to the dietary conditions after weaning.

Leliveld et al. (2013) studied the effect of wean age (21, 28, and 35 d) on post-weaning of *Escherichia coli* concentrations until 10 weeks of age. Pigs weaned at 21 d had higher counts of *E. coli* than those weaned at 28 d. Stressors factors associated to undigested nutrients in the GIT promotes fermentation and proliferation of pathogenic bacteria such as *E. coli* (ROSELLI et al., 2017).

2.2 Intestinal immune system

As described before, GIT is the first barrier against the luminal environment to protect animals from pathogenic microorganisms and antigenic substances found in the feed. When pigs experience periods of challenge, such as diseases or stress triggering to low feed intake, the integrity and function of the GIT are compromised, and it becomes more susceptible to pathogens translocation. Besides digestion, absorption, and endocrine functions, the GIT has immune functions, being considered the largest organ of the immune system.

The maintenance of intestinal homeostasis is essential for the development of the neonatal pig, but it also will certainly have important effects for health and performance throughout the productive life of the animal. Understanding the mucosal immunity and how it applies to the inductive and effector sites is particularly important in piglets because of the development of GALT occurs as the pig grows (BURKEY et al., 2009).

A highly activated immune system may seem to be ideal as a protective mechanism for animals exposed to large number of antigens, as commonly occurs in intensive animal production systems. However, stimulation of the immune system can have a substantial negative effect on the performance of animals, particularly when exposed to weaning challenges (PLUSKE, 2018).

The immune system is considered a complex comprising three major defense mechanisms: external barriers including physical (mucosal membranes) and chemical (defensive peptides and mucus) barriers; innate immune response; and adaptive immune responses (MOSER and LEO, 2010).

The immune system is made up of entire organs and lymph vessels, but also of individual cells and proteins. Lymphoid organs are classified as primary lymphoid organs such as the thymus and bone marrow. Secondary lymphoid organs such as lymph nodes, spleen, tonsils, mucosal- and gut-associated lymphoid tissues (MALT and GALT, respectively) is where expansion of lymphocytes after antigen exposure takes place, as well as the production of memory T cells and effectors B cells. Finally, the tertiary lymphoid tissues are aggregations of lymphoid cells in autoimmune diseases (PABST, 2007).

Because of the large surface area of the GIT in addition to the continuous exposure to commensal and pathogenic microorganisms makes mucosal immunity subject of interest for the last decades. The GALT execute its function by three major pathways: forming a physical barrier that prevents the pathogens and/or toxic compounds from entering the mucosa and systemic circulation (PIÉ et al., 2004); mediating subsequent innate and adaptive immune responses after activation (BURKEY et al., 2009); and last but equally important, achieving a homeostatic balance between tolerance and immune response (ARTIS, 2008).

The appendix, mesenteric lymph nodes (MLNs), isolated lymphoid follicles, and Peyer's patches (PP) are the inductive sites with areas of antigen sampling located in the mucosa. In general, the immune cells that are in the lamina propria and epithelium but not in the PP constitutes the effector site (BRANDTZAEG and PABST, 2004). In the effector sites are located selected cells of the intestinal epithelium, such as intraepithelial lymphocytes (IEL), Goblet cells, Paneth cells, enterocytes (WERSHIL et al., 2008), and lymphocytes in differentiation defending the organism in an immune response.

Intestinal epithelial cells (IEC) separate luminal contents of the GIT from mucosa tissues as intrinsic mechanism forming a physical barrier (YEN and WRIGHT, 2006), but also have a role activating and driving the host mucosal immune responses. IEC express PRRs, including Toll-like receptor 1 to 10 (TLR), and nucleotide oligomerization domain 2 (NOD2), as well as secretes cytokines and chemokines (McDERMOTT and HUFFNAGLE, 2013). This capacity of PRRs expression is the mechanism of the IEC to recognize and differentiate the commensal-associated molecular patterns and PAMPs. The stimulation to secret immunological mediators, the immunosurveillance, and what targets for immune effectors act are established and driven through communication among the lumen, IEC, and the lamina propria (BURKEY et al., 2009).

Recognition and differentiation of PAMPs by TLR initiates a cascade that results the activation of nuclear factor-κB (NF-κB) and transcription of proinflammatory cytokines. Like

cytokine and chemokines mediators, the expression of antimicrobial peptides (AMPs) has been described to be related to signaling via TLR (PETERSON and ARTIS, 2014).

As a primary function, the physical barrier formed by IEC is sustained by the establishment of the tight junctions. The tight junction not only form a selective layer but also establishes the apical and basolateral domains through polarization. Moreover, the barrier function is reinforced by the presence of AMPs and a layer of mucin covering the mucosa (BURKEY et al., 2009).

IEC includes enteroendocrine cells, goblet cells and Paneth cells. The link between the central and enteric nervous systems are done by enteroendocrine and mucosal neurons cells systems secreting hormones that regulates the digestive (CHESNÉ et al., 2019). On other hand, to establish the physical and biochemical barrier, Goblet cells secrete highly glycosylated mucins (MUC). Enterocytes and Paneth cells secretes AMPs such as defensins, cathelicidins and lysozyme. Thus, the combined functions of IEC decrease the number of microorganisms reaching and/or interacting with the mucosa (DUPONT et al., 2014).

Intraepithelial lymphocytes (IEL) are another cell type, subclass of T cells, present in the epithelium. They can be αβ and γδ T-cell receptor positive, with the γδ+ being important in young animals. In human and murine, IEL have immune regulatory properties like the maintenance of mucosa integrity and protection from pathogens. The difference between IEL and peripheral T lymphocytes is the fact that they are CD8+ whereas the others are CD4+ and CD8+ T-cell (BURKEY et al., 2009). Being very close to the lumen and able to quickly initiate inflammatory response and express protection signals, IEL are known as a key first-line guards in the GALT (PETERSON and ARTIS, 2014). The number of IEL significantly increase in human patients with celiac disease as a response to constantly and exacerbated inflammatory response in the GIT (Cukrowska et al., 2017). In young pigs, IEL are recruited to the small intestine because of weaning challenge which make them a marker of degree of inflammation at this time (Ren et al., 2015).

2.3 Feed additives

As the demand for meat globally increased in the last decades antibiotics have been used as growth promoters (AGP) in the diets of pigs and other farm animals to improve production efficiency. These compounds in the feed can improve feed efficiency, growth performance, and reduce the consequences of some subclinical illness observed as morbidity

and mortality. Improvements around 4 to 8% in average daily gain, and in between 2 to 5% for feed efficiency with their inclusion in the feed (EWING and COLE, 1994).

Feighner and Dashkevicz (1987) proposed four hypotheses to describe AGP mode of action: 1) less nutrient utilization by bacteria; 2) improvement in nutrient absorption due to a thinner layer of the mucosa; 3) lowering the amount of bacterial toxins in the intestinal lumen; and 4) reducing the incidence of subclinical diseases compromising the GIT.

Butaye et al. (2003) described the mainly concerns around the antibiotic use in animal production, which raised from the idea that this practice can generate resistance in bacteria of human and animal origin, such as *Salmonella* spp. and *Escherichia coli*. Therefore, some recommendations of restricting their use to those that promotes health but have minimum application for humans as therapy and must not promotes bacterial resistance (United Kingdom, Swann Report, 1969). Specifically, it was recommended that the use of penicillin, tetracycline, tylosin, and sulfonamides as growth promoters be discontinued because of their importance as agents in human therapy.

Daeseleire et al. (2016) describes some of the mechanisms of bacterial resistance to antibiotics. One of them is through degradation and/or modification of the antibiotic agent. They are also able to modify the cellular target through mutations as well as cell wall permeability resulting in lowering the intracellular concentration of the antibiotic agent. Finally, they can create different enzymatic pathways to avoid the drug mode of action (DAESELEIRE et al., 2016).

Feed and water additives have been applied as an alternative to the use of AGP, what is one of the most important concerns on global livestock production. According to the World Health Organization (WHO) (FAO, 2012) report, the bacterial resistance to antibiotics should be treated as a worldwide risk like climate change and terrorism. The follow sections present selected nutritional additives used in nursery diets focusing on main characteristics, mode of action, effect on gut immune response, and effects on pig growth performance.

2.3.1 Acidifiers

Acidifiers are often used as alternatives to AGP because of their ability to modify the intestinal environment in favor of beneficial microorganisms which results in enhanced nutrient digestibility, greater growth performance while reducing incidence of diarrhea. There are three ways to provide acids: organic acids, inorganic acids, and salt of acids.

The most common organic acids used as feed additives are benzoic, lactic, formic, fumaric, and citric acids. Inorganic sources of acids such as hydrochloric, sulfuric, and phosphoric acids are also commonly used as feed additives as well as some salts like calcium-formate, potassium-diformate, sodium-diformate, and sodium-fumarate (LIU et al., 2018).

Before weaning, the stomachal pH is around 4 to coagulate milk proteins. However, after weaning, a maximum pepsin activity is desired for vegetable sources be digested, and that happens at the pH of 2-3.5. Lowering the GIT pH using acidifiers produces different benefits in the gut such as greater pepsin activity, which ultimately improves protein digestion, as well as greater overall nutrient digestibility because of improvements in the mucosa integrity (SUIRYANRAYNA and RAMANA, 2015).

Different organic acids or their combination forming blends have been used in the diet of young pigs. Some of the mode of actions of organic acids in intestinal health and development were discussed by de Lange et al. (2010): the lower pH in the GIT and enzymes secretion stimulation favoring the digestion and absorption of nutrients, especially proteins; the non-dissociated forms of organic acids acting as antimicrobial agents. Moreover, the organic acids are a quick energy source for intestinal epithelial cells, especially butyric acid (SUIRYANRAYNA and RAMANA, 2015).

The antimicrobial activity of organic acids is explained by their penetration into the bacterial cell and disrupting the intracellular metabolism, mainly lowering enzymatic activities due to the lower pH, causing bacterial death and/or inhibiting their growth (SUIRYANRAYNA and RAMANA, 2015). Given that, it is expected that organic acids also have an indirect effect on gut immune response (Table 1).

Table 1 – Acidifier's mechanisms of how impact the immune response in the GIT of pigs.

Mechanism	Reference
Decreased TNF-α and increased IgG concentration in the plasma	Kuang et al. (2015)
Decreased TNF-α and increased TGF-β expression in jejunum	Kuang et al. (2015)
Increased content of serum IgG and IgA	Han et al. (2018)
Increased content of serum IgM and IgG	Long et al. (2017)
Na-butyrate downregulate inflammatory cytokines from macrophages after exposure to *Escherichia coli* LPS	Fukae et al. (2005)
Increased presence of goblet cells in colon	Manzanilla et al. (2006)

In addition to the antimicrobial activity of organic acids and their capacity of lowering pH and enhance digestion, Verstegen and Williams, (2002) suggested that they also can

preserve the feed. Therefore, acidifiers have be shown to be a potential alternative to the use of antibiotics as growth promoters in nursery diets due its properties of promoting gut health and consequent better growth performance. Compiled results of growth performance of weaned pigs fed with different sources of acidifiers are shown in Table 2.

Table 2 – Effect of acidifiers on average daily gain (ADG), feed conversion ratio (FCR), and average daily feed intake (ADFI) of nursery pigs[1].

Item	ADG	FCR	ADFI	References
Citric acid	(+)	NS	(+)	Suiryanrayna et al. (2012)
Citric acid	NS	NS	NS	Radcliffe et al. (1998)
Formic acid	NS	(+)	NS	Manzanilla et al. (2004)
K-diformate	(+)	(+)	NS	Kluge et al. (2006)
Ca-formate	(+)	(+)	NS	Bosi et al. (2007)
Na-butyrate	NS	(+)	NS	Manzanilla et al. (2006)
Na-butyrate	NS	NS	NS	Weber and Kerr (2008)
Fumaric acid	(+)	(+)	(+)	Lawlor et al. (2005)
Fumaric acid	NS	NS	NS	Kil et al. (2006)
Benzoic acid	NS	NS	NS	Kluge et al. (2006)
Benzoic acid	(+)	(+)	(+)	Torrallardona et al. (2007)
Benzoic acid	(+)	(+)	NS	Guggenbuhl et al. (2007)
Blend[2]	(+)	(+)	NS	Han et al. (2018)
Blend[3]	NS	NS	NS	Yang et al. (2019)
Blend[4]	(+)	NS	NS	Grilli et al. (2010)
Blend[4]	(+)	(+)	NS	Li et al. (2008)
Blend[5]	NS	NS	NS	Li et al. (2008)
Blend[6]	NS	NS	NS	Walsh et al. (2012)
Blend[7]	(+)	(+)	(+)	Kuang et al. (2015)
Blend[8]	(+)	(+)	NS	Long et al. (2017)
Blend[9]	(-)	NS	(-)	Ahmed et al. (2014)

[1](+): significantly improved; (-): significantly decreased; NS: non-significant;
[2] Blend of formic, acetic, lactic, propionic, citric, and sorbic acids with their ammonium salts.
[3] Blend of fumaric acid, citric acid, malic acid, and MCFA (capric and caprylic acid).
[4] Blend of butanoic acid, fumaric acid, and benzoic acid.
[5] Blend of citric acid and sorbic acid.
[6] Blend of propionic acid, acetic acid, and benzoic acid.
[7] Blend of fumaric Ca-formate, Ca-lactate, citric acid and MCFA (lauric, myristic and capric).
[8] Blend of formic acid, propionic acid, and acetic acid.
[9] Blend of formic acid, propionic acid, lactic acid, and phosphoric acid.

As in other feed additives, the development of bacterial resistance to organic acids is a concern as it was described through proteins resistant to acids lower than 2.5 by Sato et al. (2000) in *E. coli* and in *Salmonella typhimurium* by Bang et al. (2000). Another concern regarding acidifiers utilization in the feed or water, particularly in the weaned pigs, is that pH

below 4.8 in the feed may have negative impact the voluntary feed intake (VERSTEGEN and WILLIAMS, 2002).

2.3.2 Zinc

Zinc (Zn) is a trace mineral required in several cellular functions such as replication, transcription, and signal transduction. It is a component and activator of more than 300 metalloenzymes and has an important role on hormones secretion. Furthermore, it plays a role on intestinal barrier integrity, and regulation of immunological responses (McDOWELL, 1992, SALES, 2013). Zinc requirements for young pigs are 46.8 mg/d for 7 to 11 kg and 72.4 mg/d for 11 to 25 kg of BW (NRC, 2012). The inclusion of pharmacological doses (1500 – 3000 ppm) of zinc oxide in the diet of young pigs to reduce post-weaning diarrhea and promote growth is a common practice (POULSEN, 1998; SMITH et al., 1997; HILL et al., 2000).

Liu et al. (2018) reviewed the possible mode of action of pharmacological doses of zinc oxide: improvements in intestinal histomorphometry, promoting cellular regeneration after epithelial damage, stabilizing the microbiota and diversity of the coliform microbes, and promoting lymphocyte proliferation. High levels of Zn also reduce the intestinal permeability by increasing the up regulation of tight junctions, such as occludin, claudin-1, and ZO-1 (HU et al., 2013; ZHU et al., 2017).

The dietary inclusion of ZnO also demonstrated to decrease diarrhea incidence and down regulate proinflammatory cytokines (HU et al., 2013). Besides its action on intestinal barrier function and ameliorating inflammation induced by cytokines in the post-weaning period, Zhu et al. (2017) showed an improvement of plasma antioxidant capacity of weaned pigs. It also improves feed intake through increased ghrelin production (YIN et al., 2009). However, according to Debski (2016) Zn mode of action on controlling diarrhea is still not fully understood.

All these different mechanisms that high levels of Zn on diets of pigs promotes intestinal health were reviewed by Roselli et al. (2003). Nutrient absorption is reduced during diarrhea, due to losses in surface area for absorption, then, Zn may reduce its bioavailability. Given that, Zn can be insufficient for mucosal repairment followed by diarrhea occurrence, which is ameliorate by supplementation of high levels of zinc in the diet.

Given the role in decreasing bacteria adhesion to intestinal cells (Roselli et al., 2003) and the capacity to down regulate the proinflammatory response because of this mode of

action, the overall result of zinc is the enhancing of gut barrier function, since proinflammatory cytokine are directly related to the down regulation of tight junctions (Table 3). Zhang and Guo (2009) studied the occludin, ZO-1 and claudin-1 expression in the ileum of weaned pigs supplemented with different sources of zinc. The result indicated that high dietary levels of Zn up regulated both mRNA and protein expression of occludin and ZO-1.

Table 3 – Pharmacological doses of zinc mechanisms of how impact the immune response in the GIT of pigs.

Mechanism	Reference
Decreased TNF-α, INF-γ, and IL-6 expression in jejunum.	Hu et al. 92013)
Decreased IL-1, INF-γ, and increased TGF-β expression in jejunum.	Zhu et al. (2017)
Increased Goblet cells in jejunum villous.	Zhu et al. (2017)
Decreased TNF-α and INF-γ expression in ileum.	Grilli et al. (2015)
Decreased TNF-α, IL-8, and increased TGF-β in Caco-2 cells infected with ETEC.	Roselli et al. (2003)

Hill et al. (2001) demonstrated that zinc oxide supplementation to young pigs improved growth performance at dietary inclusion of 1,500 to 2,000 mg Zn/kg. Earlier-weaned pigs, before 15 d of age, may benefit more from pharmacological doses of zinc oxide, but the good response is also seen in pigs older than 21 d. Sales (2013) concluded in a meta-analysis that pharmacological doses of zinc oxide increased growth of pigs during the post-weaning phase. Compiled results of growth performance of weaned pigs fed with different doses and sources of Zn are shown in Table 4.

Table 4 – Effect of pharmacological doses of zinc on average daily gain (ADG), feed conversion ratio (FCR), and average daily feed intake (ADFI) of nursery pigs[1].

Item	ADG	FCR	ADFI	References
ZnO, 2250 ppm	(+)	NS	(+)	Hu et al. 92013)
ZnO, 3000 ppm	(+)	NS	NS	Smith et al. (1997)
ZnO, 3000 ppm	(+)	NS	NS	Zhu et al. (2017)
ZnO, 3000 ppm	(+)	(+)	NS	Grilli et al. (2015)
ZnO, 2500 ppm	(+)	NS	NS	Poulsen (1995)
ZnO, 3000 ppm	(+)	(+)	(+)	Hill et al. (2000)
ZnO, 3000 ppm	(+)	(+)	(+)	Hill et al. (2001)
ZnO, 3000 ppm	(+)	NS	(+)	Namkung et al. (2006)
ZnO, 3000 ppm	(+)	(+)	(+)	Pettigrew (2006)
ZnO, 2000 ppm	(+)	(+)	(+)	Zhang and Guo (2009)
ZnO, 3000 ppm	(+)	NS	(+)	Shelton et al. (2011)
ZnO, 3000 ppm[3]	(+)	NS	(+)	Case and Carlson (2002)
ZnO, 2500 ppm	(+)	NS	(+)	Feldpausch et al. (2018)

ZnO, 3000 ppm	(+)	(+)	(+)	Pérez et al. (2011)
ZnO, 3000 ppm	(+)	(+)	(+)	Woodworth et al. (2005)
ZnO[4]	(+)	(+)	(+)	Sales (2013)
Ds-ZnO, 500 ppm[4]	NS	NS	NS	Song et al. (2015)

[1](+): significantly improved; (-): significantly decreased; NS: non-significant;
[2] Same response was obtained for pigs weaned before 15 d and after 21 d of age.
[3] Inorganic source of zinc, 3000 ppm versus inorganic source of zinc, 150 ppm.
[4] Meta-analysis, Sales, 2013.
[5] Ds-ZnO = mixture of diosmectite (DS) and ZnO.

Besides the positive effects of feeding pharmacological doses of zinc to nursery pigs, this practice has some important concerns, such as antimicrobial resistance, environmental issues, and loses in digestibility of other minerals. The digestibility of Ca and P as well as the efficacy of phytase in the diets of young pigs were negatively affected by pharmacological levels of ZnO (Walk et al., 2015). In the European Union, restrictions, and discontinuation of inclusion of ZnO will be happening in 2022 whereas other regions of the world will still be using this practice (LIU et al., 2018).

2.3.3 Copper

Like discussed above for zinc, copper is also essential for several enzymatic processes and metabolic reactions (LIU et al., 2018). Newborn pigs require around 5 to 6 mg/kg of Cu for normal physiological functions and metabolism (NRC, 2012). Pigs deprived of copper develop critical dysfunctions, microcytic anemia, and bone abnormalities. As the same as zinc, inclusion of pharmacological levels of Cu (100 – 250 ppm), mostly in sulfate form, in the diet of young pigs is a common practice to improve growth and lowering diarrhea incidence (LIU et al., 2018).

Pharmacological levels of Cu may positively impact microbiota due to its bacteriostatic and bactericidal properties (DEBSKI, 2016). It has also been suggested that feed intake improvements because of Cu supplementation is due to its ability to up regulate the mRNA expression of neuropeptide Y (LIU et al., 2018).

Lymphocyte proliferation may not be affected by pharmacological levels of copper in non-challenged nursery pigs (20 kg of BW; DAVIS et al., 2002). The lack of direct effect on GALT immune response due zinc supplementation is also valid for copper supplementation. Namkung et al. (2006) did not find any difference on zinc or copper supplementation on cytokines (INF-γ, IL-1β and TNF-α) and cortisol concentration in the plasma of pigs. Also, no interactions between diet and a LPS challenge were found. Yet, zinc and copper supplementation reduced the diversity of ileal microbiota, while copper decreased the diversity of microbiota in the colon.

Commercial diets for weaned pigs generally contain pharmacological doses of copper in between 150 - 250 mg/kg. Bikker et al. (2016) demonstrated that copper ($CuSO_4$, 160 mg/kg) supplementation significantly improved growth and fecal consistency in young pigs. Compiled results of growth performance of weaned pigs fed with different doses and sources of copper are shown in Table 5.

Table 5 – Effect of pharmacological doses of copper on average daily gain (ADG), feed conversion ratio (FCR), and average daily feed intake (ADFI) of nursery pigs[1].

Item	ADG	FCR	ADFI	References
Cu- AA, 100 ppm	(+)	NS	(+)	Pettigrew (2006)
$CuSO_4$, 100 ppm	(+)	(+)	NS	Pérez et al. (2011)
$CuSO_4$, 125 ppm	(+)	NS	(+)	Feldpausch et al. (2018)
Cu-Lys, 200 ppm	(+)	(+)	(+)	Apgar et al. (1995)
Cu-Lys, 250 ppm	(+)	NS	(+)	Namkung et al. (2006)
$CuSO_4$, 250 ppm	(+)	(+)	NS	Hill et al. (2000)
$CuSO_4$, 150 ppm	(+)	NS	(+)	Shelton et al. (2011)
$CuSO_4$, 250 ppm	(+)	NS	(+)	Smith et al. (1997)
$CuSO_4$, 250 ppm	(+)	NS	(+)	Stahly et al. (1980)
$CuSO_4$, 250 ppm	(+)	NS	(+)	Veum et al. (2004)
Cu-proteinate, 200 ppm	(+)	NS	(+)	Veum et al. (2004)
$CuSO_4$, 225 ppm	(+)	NS	(+)	Fry et al. (2012)
Tribasic Cu chloride, 225 ppm	(+)	NS	(+)	Fry et al. (2012)
$CuSO_4$, 160 ppm	(+)	(+)	(+)	Bikker et al. (2016)
$CuSO_4$, 175 ppm	(+)	NS	(+)	Davis et al. (2002)

[1](+): significantly improved; (-): significantly decreased; NS: non-significant;
[2] Same response was obtained for pigs weaned before 15 d and after 21 d of age.

Synergetic effect of pharmacological levels of Cu and Zn has demonstrated divergent results. Some researchers have showed no synergic effects using both minerals at pharmacological levels (250 mg/kg of Cu, 3,000 mg/kg of ZnO; HILL et al., 2000). Yet, other studies have showed the opposite, where growth performance was improved by feeding both mineral at pharmacological levels (125 mg/kg of Cu, 3,000 mg/kg of ZnO; SHELTON et al., 2011) or feeding Cu in organic or inorganic forms in combination with 3,000 ppm Zn (PÉREZ et al., 2011). Nevertheless, both zinc and copper mechanism of action remain to be fully elucidated.

The undesirable negative impact of feeding great concentrations of copper is its high fecal excretion, increasing fourteen times when 250 ppm Cu as CuSO4 is included in the diet (VEUM et al., 2004). The fact is explained by the extremely low retention of Cu (1.1 mg/kg BW) and results in majority of what was fed being excreted into the environment (BIKKER et

al., 2016). Great excretions of Cu and Zn on environment can results in toxic effects to plants and microorganisms in the soil, calling attention in some areas of intensive swine production.

2.4 Mycotoxins

Mycotoxins are a worldwide concern in pork production as they affect especially young animals and those at reproduction phase. Most feedstuffs used in the pig`s diet such as corn and grain by-products are susceptible to be contaminated with these secondary fungal metabolites which causes toxicity and growth retardation in pigs (Harper et al., 2010). The four major types of mycotoxins affecting pigs are zearalenone (ZEA), deoxynivalenol (DON), fumonisin (FUMO), and aflatoxins (AFLA).

On reproduction, ZEA impacts the oocyte quality (Xu et al., 2020), decreases number of pigs born (Zhang et al., 2015), prolongs wean to estrus interval, and increases embryonic mortality rate (Young et al., 1990). Bouhet et al. (2004) showed that FUMO impair the intestinal barrier function through decreasing the transepithelial electrical resistance and cell proliferation in an *in vitro* study. Deoxynivalenol (Holanda and Kim, 2020) and AFLA (Harper et al., 2010) often negatively affect growth performance of pigs in the nursery phase as it largely damages the liver and its functionality. Therefore, mycotoxins may impair the reproduction, hepatic, intestinal, and immunological functions in pigs (Lindeman et al., 1993).

Mycotoxicosis ameliorators are important in pig`s diet as some mycotoxins effects are often subclinical as shown by reproduction failures and slowing growth. Mycotoxin binders are commonly used in the feed as they mitigate the negative effects with compounds such as hydrated sodium calcium aluminosilicate (HSCA; Xu et al., 2020; Harper et al., 2010).

The mode of action of mycotoxins binders still controversial in the literature. Yet, some mechanisms have been discussed: selective chemisorption, electron donating, hydrogen bonding, furan ring bonding, ion interactions and coordination between exchange cations and the carbonyl groups (Elliot et al., 2020).

First studies using HSCA were done by Harvey et al., (1989) looking at its effects on pig performance and later comparing two different sources of HSCA (Harvey et al., 1994) on amelioration of induced-aflatoxicosis in growing pigs' performance. The effects of HSCA were evaluated by Xu et al. (2020) on amelioration of ZEA-contaminated diets on oocytes quality. It was observed that HSCA in the diet can reduce effects of ZEA and improve oocytes quality in sows. However, the impact of DON in the diets of finishing pigs were not affected by the inclusion of HSCA as no effects on growth (Patterson and Young, 1993) nor carcass

traits were observed (Matthews et al., 1999). In early stages of the nursery phase, Richert et al. (2021) observed that a commercial product classified as HSCA, improved ADG and ADFI in phase 2 (27 -34 d old) and better feed efficiency at phase 3 (35 – 42 d old) of pigs fed diets containing common levels of mycotoxin in the USA Midwest.

The undesirable effects of mycotoxin binders include cytotoxic effects including oxidative stress, reduction in cell viability, apoptosis, and DNA damage. They can bind essential micronutrients and vitamins. Moreover, they can interact with veterinary drugs, which may cause a decline or an increase in the oral absorption of drugs, leading to a potential therapy failure. Mineral adsorbents may also contain variable amounts of accessory minerals (quarts, nontronite, erionite), heavy metals (lead, copper, cadmium), dioxins, and trace elements, which can induce toxicity in livestock animals as well as alter serum mineral profile and activities of enzymes (Elliot et al., 2020).

3 FINAL CONSIDERATIONS

It is unlikely that there is any single feed additive or combination that could replace the function of feed antibiotics as growth promoters (AGPs) with the same expected outcome. Furthermore, any feed or water additive used with the purpose to fully replace the role of antibiotics in livestock diets will be subject to the same intense and careful examination that antibiotics have been subjected. As described in this review, the use of antibiotics has consolidated results in pig performance and health through many different effects on the GIT. Then, the success of any strategies regarding the replacement of AGPs in pig diets will depend on a combination of nutritional, management, housing, biosecurity, and health factors.

Another factor to be improved is the inconsistency in the experimental studies of the many alternatives evaluated for pigs, which makes it difficult to judge the efficacy of a particular additive. Also, some additives lack results in commercial farms, which is the ultimate information regarding the applicability of such technology rather than highly controlled conditions found in most experiments. Yet, to find such “silver bullet”, the pig industry has been growing and many companies created, which make this market very interesting for researchers.

The microbiota-enteric nervous and immune system axis, not in a particular order, might be the key for all questions regarding animal health and growth. Understanding the GALT mechanisms and its interrelation with the commensal microbiota and the nervous system can give us insights on how to better approach and make interventions to promote gut health. As described in this review, most of the feed additives and ingredients might have either a direct immune modulation effect on up or down-regulating gut immune response or indirect contributions to the immune homeostasis, for example, promoting microbiome equilibrium.

It can be suggested that the microbiome equilibrium is the factor that might trigger all the other events in the gut. Thus, all strategies that are proved to promote this balance are important. In this regard, organic acids, despite the contradictory of results in performance, might be important players to manipulate the GIT microbiota to maintain animal gut health, through diversity, stability, metabolites, and crosstalk with the epithelium and the GALT. Nevertheless, the use of these substances should be considered as prevention of GIT disturbs much more than a therapeutic approach.

More consistent than the alternatives cited above, the use of pharmacological doses of zinc and/or copper has showed better results in growth performance, sometimes at the same magnitude of some AGPs. However, such substances have no long potential to be the perfect replacer of AGPs, because of the possibility of antimicrobial resistance and environmental issues, zinc had its use restricted in many countries already. Yet, just like AGPs, while pig industry waits for such ban, zinc oxide might be the most effective alternative to replace AGPs in post-weaning diets from the standpoint of growth performance results.

The utilization of mycotoxins binders in nursery diets is an important practice that swine nutritionist applies in the field. Feedstuffs are constantly varying in terms of nutritional composition and levels of contaminants. Mycotoxins, in most cases, are present in grains and grain by-products and their concentration vary among season, storage, and crops technification levels. Yet even though there is lack in the literature of minimum and maximum levels of mycotoxins that can impact growth and health, mycotoxin binders are included regardless of levels found in the ingredients. Also, its utilization might be easily combined with other additives, such as organic acids, probiotics, and prebiotics.

A combination of different approaches may provide the most effective alternative to AGPs, considering different mode and site of actions, synergism effects, microbiota stability, and the combination of specific and non-specific targets to avoid bacterial mechanisms of resistance.

Finally, despite any scientific advances regarding the development of new substance or the improvement of those that are in current use, one of the best options to achieve success in the nursery phase, always will be the proper attention for basic principles in the diet formulation and pig management. Post-weaning diets always will require high-quality and more processed ingredients, proper form of presentation, good palatability, and last but not least, high quality drinking water. Also, attention to biosecurity procedures, such as vaccination and facilities disinfection, does no doubt enhance pig health and diminish disease challenges. Additionally, efforts in the pre-weaning period must be another way to have a healthy pig at the nursery phase. In this regard, early life interventions may be considered and further investigated, such as after-birth gut colonization and development, colostrum intake, and weaning age, and sow treatments that change piglet colonization during lactation.

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CHAPTER 2

Article - Effects of organic acids in the water for weaned pigs on growth performance, behavior, and intestinal health

***Translational Animal Science* formatting guidelines –** Submitted version

Additives for weaned pigs

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ABSTRACT: The objective of this research was to investigate the effect of using organic acids (OA) in the drinking water on performance and general health of nursery pigs over a 41 d supplementation period as an alternative to therapeutic antibiotics in the feed. One hundred-twelve weaned pigs (5.17 kg, approximately 20 d of age, barrows) were assigned in a completely randomized block design with four treatments and seven replicates: negative control (NC, basal diet), positive control (PC, NC + 150/120/80/80 mg/kg of colistin in each of four phases, respectively), and two doses of organic acids, OA1 (1.0 mL OA/L of water),

and OA2 (2.0 mL OA/L of water). Twice a day, the daily water usage/pen was recorded, and the fecal score analyzed to calculate the diarrhea incidence. At days 3 and 4 all pigs were orally inoculated with *Escherichia coli* F4 (1mL; 1 x 10^7 CFU/mL) to promote an experimental infection and an immunological challenge. On day 9, 28 pigs (one per pen closest to the pen mean BW) were euthanized for collection of jejunum tissue (evaluation of mucosal integrity, and quantification of antioxidant enzymes), and cecum contents for volatile fatty acids (VFA) analysis and *Bifidobacterium* and *Lactobacillus* CFU/g count. No differences among treatments were found for growth performance ($P > 0.05$) during any period of the study. The inclusion of OA in the water (OA2) increased daily water usage when compared to NC and PC for all weeks of evaluation and for the overall period ($P < 0.05$). No statistical difference was detected for diarrhea incidence. No differences were found for mucosal integrity and CFU/g in cecal contents ($P > 0.05$). OA1 tended to have greater concentration of acetic, propionic acid, and total VFA concentration when compared to OA2 and did not differ from NC and PC ($P < 0.10$). OA1 had the greatest concentration of jejunal superoxide dismutase ($P < 0.0001$). NC had the least and PC the greatest concentration of catalase with OA1 and OA2 being intermediates ($P = 0.037$). OA1 tended to have greater concentration of glutathione transferases when compared to PC ($P = 0.094$). No differences were found for glutathione and lipid peroxidation in the jejunum ($P > 0.05$). Organic acids in the drinking water for weaned pigs, as an alternative to colistin, improve daily water usage and the antioxidant capacity in the jejunum.

Keywords: antioxidant enzymes; diarrhea; drinking water; piglet.

List of abbreviations: CAT, catalase; DWU, daily water usage; GIT, gastrointestinal tract; GST, glutathione-S-transferases; OA, organic acids; ROS, reactive oxygen species; SID, standardized ileal digestible; SOD, superoxide dismutase; VFA, volatile fatty acids; WBC, white blood cells.

INTRODUCTION

After birth, the gastrointestinal tract (GIT) of pigs undergoes maturation processes including changes in size (Lindemann et al., 1986), barrier function (Takeda et al., 2004), enzymatic secretion (LE Huërou-Luron et al., 2002), microbiota colonization (Kubasova et al., 2018), and immunological functions (Bailey et al., 2005; Wilson et al., 2005). At weaning, pigs are subjected to an abrupt separation from the sow, transportation, social

hierarchy stress, different environment, and increased exposure to pathogens and dietary antigens. The pigs must adapt to all these stressors to be productive and efficient (Campbell et al., 2013). The GIT development during early life stages is a plastic process, so it can be modified by environmental stimulus. Thus, the weaning stressors events might define the GIT development and its functions (Moeser et al., 2017). The GIT comprises several layers of defense mechanisms that, acting together, control normal functions of the gut while protecting the host from luminal contents (Moeser et al., 2017), which includes pathogens and antigenic compounds. Therefore, it plays a central role in the overall health of pigs. Disruption of GIT barrier function is a central cause for much of the enteric issues in post-weaned pigs (McLamb et al., 2013); it is marked by local and systemic pro-inflammatory responses (Pié et al., 2004) and increasing production of reactive oxygen species (ROS; Wang et al., 2017). Hence, weaning is associated with an increase in oxidative stress, which is defined by a decline in antioxidant enzyme activity and oxidative injuries of proteins, DNA, and lipids in multiple body tissues, including the intestine (Yin et al., 2014).

The use of antibiotics as growth promoters in pig's diet due to their effects on controlling subclinical diseases and thereby promoting growth (Cromwell, 2002) has been globally discouraged. Colistin has been extensively used in the feed as growth promoter for the control of enteric infections in pigs. Yet, livestock animals have been charged as the principal reservoir for colistin resistance amplification and spread (Rhouma et al., 2016). Thus, the use of colistin in animal feed was banned in 2016 in many countries as growth promoter but is available for therapeutic purposes. Therefore, the implementation of sustainable alternatives for enteric infection prevention should be actively encouraged in pig production (Rhouma et al., 2016).

Organic acids (OA) are known to have a positive effect on growth performance due to their capacity to reduce the luminal pH and their antimicrobial action (Suiryanrayna and Ramana, 2015). Lowering luminal pH, especially in the stomach, enhances protein digestion, decreases stomach emptying rate, stimulates enzyme production (de Lange, et al., 2010), and decreases diarrhea incidence (Torrallardona et al., 2007). Mechanistically, the undissociated forms of OA can penetrate the bacterial cell wall, dissociate into anions and protons disrupting bacterial metabolism (Verstegen and Williams, 2002), and then inhibit pathogen proliferation (Diao et al., 2014). Up to now, OA have been delivered to pigs in the feed or in the water as additives in the form of blends or single OA. Indeed, providing OA in the drinking water to weaned pigs seems a better option. There is more flexibility in adjusting

dosage and the amount of OA that can reach the GIT when compared to OA in the feed, water delivered OA can be greater considering that pigs drink at least twice the amount they eat (Escudero et al., 2016). Therefore, we hypothesized that providing OA in the drinking water can alleviate the consequences of weaning stressors through enhancements in intestinal health parameters, such as antioxidant capacity. The objective of this research was to investigate the effect of using OA in the drinking water on growth performance and general health of nursery pigs, as an alternative to the use of antibiotics in the diet.

MATERIALS E METHODS

All experimental procedures and animal housing for the present study were approved by the Ethics Committee on Animal Use of the Federal University of Lavras (CEUA) under the Protocol 048-17. The experiment was performed at nursery facilities of the Experimental Swine Center at Federal University of Lavras, Brazil. The OA blend was provided by Trouw Nutrition (Campinas, Brazil).

Animals and treatments

A total of 112 barrows (DB90×PIC337) were weaned (approximately 20 d of age), individually weighed, and allotted to the treatments based on initial BW (5.17 ± 0.43 kg). Pigs were housed in fully plastic slatted floor pens (0.34 m^2/pig) with a galvanized steel feeder and a single nipple waterer. They were assigned to one of four treatments with seven pens per treatment (4 pigs/pen). Pigs were fed *ad libitum* over 41 d using four dietary phases (Table 1) based on industry recommendation formulations to minimize initial post-weaning nutritional stress and formulated to meet the Brazilian nutritional requirements of pigs according to Rostagno et al. (2017).

The dietary treatments included negative control (NC, basal diet), positive control [PC, basal diet + 150 (phase 1); 120 (phase 2); and 80 mg/kg (phase 3 and 4) of colistin sulfate in the feed], and two doses of OA: OA1 (1.0 mL OA/L of water), and OA2 (2.0 mL OA/L of water). For the PC diet, colistin sulfate was included at the expense of rice or corn in the basal diet. Pigs from OA treatments were given a blend of free and highly buffered OA (acetic, formic, and propionic) including copper sulfate for drinking water application. However, on weaning day, for the control of respiratory diseases, all pigs received a single dose of tulathromycin (Draxxin® Zoetis, 100 mg/ml, 0.15 mL/animal), and other individual pig therapeutic medication treatments were given and recorded according to the CEUA

guidelines. Zinc oxide is still commonly used in young pig diet around much of the world. In this study, a commercial nutritional program with zinc oxide was used to evaluate the additive effects of OA as an alternative to colistin. Pharmacological doses of zinc oxide were included in the basal diet throughout the nursery period (Phase 1, 2750; phase 2, 1950; phases 3 and 4, 1700 ppm of zinc oxide). Pigs and feeders were weighed on weaning day, days 7, 14, 21, and 41 post-weaning to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (Gain:Feed). Pigs had *ad libitum* access to water in adjustable nipple drinkers (one/pen).

Daily water usage

Individual water containers with a graduated ruler fixed next to an external transparent tube used for measuring the water column, were installed for each pen. Twice a day, the water column was recorded and the daily water usage (DWU) was calculated per pen according to the cylinder volume eq. [$V = h\pi r^2$, where V = cylinder volume (cm^3), h = water column used (cm), and r = container radius (cm)]. The water wasted by the pigs was not quantified in the present study. Water pipelines were cleaned with a broad-spectrum disinfectant with biocide action before the trial start. The OA blend was mixed in the water tanks (1 L/1000 L for OA1 and 2 L/1000 L for OA2). The pH of the water delivered in each pen was measured every week and every batch of OA mixing in the water tanks using a portable pH meter (Digimed, Sao Paulo, Brazil).

Diarrhea and belly nosing incidence

Twice a day during the experimental period, the fecal consistency was visually evaluated at the same time, by the same person, and recorded as normal (no diarrhea) or either pasty or liquid feces (presence of diarrhea) following the methods of Casey et al. (2007) throughout the experimental period. Belly nosing behavior was recorded when pigs presented repeated rhythmic up-and-down massage with the snout on another piglet's midsection (Fraser, 1978) during the first two weeks of the study. Observations for belly nosing behavior were performed from 0700 to 1000 h in the morning and 1600 to 1700 h in the afternoon. Diarrhea and belly nosing incidences were calculated as the percentage of the total number of observations per pen over the period.

Experimental infection and blood collection

At days 3 and 4 of experiment, all pigs (n = 112) were orally inoculated with 1 mL each day, with a solution of *E. coli* (10^7 CFU/mL). The bacterial inoculum used was prepared from the bacterial strain *E. coli* F4 [heat-labile toxin (LT+), heat-stable toxin a (STa+), and heat-stable toxin b (STb+)], which was validated by the Health Laboratory of São Paulo University. The strain was cultured in culture medium (Luria Bertani, Himedia) for 18 hours, separated by centrifugation and then sequentially diluted three times in PBS to a concentration of 10^7 CFU/mL bacteria according to the methodology of Halas et al. (2009).

Blood samples (one animal per pen with the similar weight of the experimental unit average, n = 28) were collected from a jugular vein at d 0, 3, and 7 in EDTA tubes (2 mL/pig) for differential leukocyte count. The WBC count (total leucocytes, neutrophils, lymphocytes, and monocytes) were determined (1000 cells/mm^3) using the Sysmex pocH-100iV Diff® hematology analyzer (Sysmex America, Inc., Lincolnshire, IL, USA), through the hydrodynamic focused impedance cell count methodology. The same pigs that had blood collected (n = 28) were euthanized by electronarcosis followed by exsanguination on day 9 of experiment for tissue harvest.

Intestinal parameters

The luminal pH of the stomach, jejunum, and ileum were measured immediately after death using a portable pH meter (Digimed, Sao Paulo, Brazil). Then, distal jejunum samples (2.0 cm) were collected for evaluation of mucosal integrity. The jejunum tissue was chosen because it is where majority of the digestion and absorption occurs. Therefore, impairment in its morphometry may result in losses in absorption with further decrease in performance. The samples were washed with saline solution, fixed in 10 % formaldehyde for 48 hours, cut in a microtome (thickness of 4 μm) and stained by hematoxylin and eosin. Ten well oriented villous and crypts were analyzed using the OLYMPUS CX31 optical microscope, with the associated OLYMPUS SC30 camera, and Axio Vision Release 4.9 (ZEISS) for evaluation of villous height and crypt depth.

Cecum content was analyzed for volatile fatty acids (VFA) concentration and microbiological population count. The culture method in a selective medium specific for *Bifidobacterium* and *Lactobacillus* spp were used. The plates were incubated at 37° C for 24 h before the counting. The colony counts (CFU/g) were done by logarithmic transformation before the statistical analysis. For VFA analysis, 2.0 g sample of the contents were added to 4

mL of formic acid (17%) to extract and preserve the fatty acids present. Centrifugation was performed at 12,500 x G for 60 minutes and the supernatant were stored at -20 ° C until analysis by gas chromatography using Agilent 7890A gas chromatograph equipped with flame ionization detector (7683B) and a fused-silica capillary column (J &W19091F-112, Agilent Technologies, Santa Clara, CA, USA) containing 0.20 μM cyanopropyl polydioxanone.

Jejunum mucosa samples were washed with saline solution and stored in liquid nitrogen for quantification of antioxidant enzymes and related products. Samples were homogenized in 0.1M potassium phosphate buffer (pH 6.5). Glutathione in the homogenate was analyzed following Sedlak and Lindsay (1968) methods which consist of a simple spectrophotometric for concomitant determination of in protein bound sulfhydryl groups various tissues where 5,5'-dithiobis- (2,-nitrobenzoic acid) is reduced by sulfhydryl groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of sulfhydryl. Lipid peroxidation in the homogenate was analyzed following Jiang et al. (1991) procedures which is based on the oxidation of Fe^{2+} mediated by hydroperoxide under acidic conditions forming a chromophore with xylenol orange which absorbs at 560 nm.

The homogenate was centrifugated, the supernatant collected and analyzed for catalase (CAT; Aebi, 1984), superoxide dismutase (SOD; Gao et al., 1998), and glutathione S-transferase (GST; Habig et al., 1974). Briefly, the reaction for CAT activity was performed in a microplate reader (240 nm), and activity measured as nmol per mg of protein. The reaction for SOD was performed in a microplate reader (440 nm) and activity measured as the ability of SOD to inhibit pyrogallol auto-oxidation (50% inhibition considered 1 SOD unit) expressed as units of SOD per mg of protein. The reaction for GST was performed in a microplate reader (340 nm) and calculated as ability of GST to conjugate the substrate 2,4-dinitrochlorobenzene with reduced glutathione, forming a thioester expressed as nmol per min per mg of protein. Results are expressed as the amount of proteins that were present in the jejunum homogenates. The tissue protein concentration was determined spectrophotometrically using the method of Bradford (1976) in a microplate reader (595nm). Antioxidant enzymes activities and concentrations were all measured in a 96-well microplate reader (Synergy HT, Biotek, VT, USA).

Statistical analysis

All variables measured were tested for normality using Shapiro-Wilk test before analysis, and any variable that failed to follow a normal distribution was transformed through the RANK procedure from SAS (SAS Inst. Inc., Cary, NC). All data were analyzed using the PROC MIXED procedure from SAS (SAS Institute Inc., Cary, NC, USA) as a randomized complete block design (initial BW). The pen was considered the experimental unit for the growth performance and DWU analysis. For the blood and intestinal health variables, each animal sampled was considered the experimental unit. All data were reported as least squares means, compared by Tukey-Kramer test, and standard errors for the means were reported. Means were considered significant different when $P \leq 0.05$ and trends were discussed when $0.05 < P < 0.10$.

RESULTS

Growth performance and water pH

Growth performance data and DWU are shown in Table 2. No difference among the treatments were observed for growth performance ($P > 0.05$) at any time period during the study. Pigs given 2 mL/L of OA in the drinking water (OA2) had greater DWU when compared to pigs that receive neither OA nor antibiotic (NC) in all periods of evaluation as well as when compared to pigs that received antibiotic (PC) and OA1 on week 2 and 3 of evaluation ($P < 0.05$). Overall, OA2 pigs had the greatest DWU among the treatments with NC having the smallest DWU and PC and OA1 being intermediates ($P = 0.0005$).

The water pH is shown in Table 3. As expected, the overall pH was lower when OA1 (3.72), and OA2 (3.28) was added in the drinking water when compared to both NC (7.04) and PC (6.98) ($P < 0.0001$).

Diarrhea and belly nosing incidence

Diarrhea and belly nosing incidences are shown in Fig. 1(a) and 1(b), respectively. No difference among the treatments were found for diarrhea and belly nosing incidence at any period or overall, during this study ($P > 0.05$).

White blood cells count

The absolute numbers of white blood cells (WBC; leukogram) in the plasma of pigs are shown in Table 4. No interaction between day of collection and treatments were found ($P > 0.05$). No effect of treatments was detected in the number of WBC in the plasma ($P > 0.05$)

expect for proportion of monocytes on d 3 when OA2 pigs had increased number of monocytes ($P = 0.022$) than all other treatments.

Intestinal morphometry, VFA, antioxidant capacity, and bacterial count

Jejunal villous height, crypt depth, and villous:crypt ratio from one pig per pen harvested on d 9 of the study are shown in Table 5. There were no differences among the treatments for jejunum morphometry ($P > 0.05$).

Volatile fatty acids concentration in cecum contents are shown in Fig. 2. Total VFA concentration tended to be greater when pigs were given 1 mL of OA/L of water (OA1) when compared to OA2, with NC and PC having intermediates values ($P = 0.0697$). The same pattern of response was found for the concentration of acetic acid ($P = 0.0969$) and propionic acid ($P = 0.0819$). Butyric, valeric, and isovaleric acids did not differ among the treatments ($P > 0.05$).

Antioxidant capacity parameters of jejunal tissue are shown in Table 6. Reduced glutathione concentration ($P = 0.8527$) and lipid peroxidation ($P = 0.2894$) in jejunum were not affected by treatments whereas GST activity tended to decrease when antibiotic was added to the diet when compared to OA1 group, with NC and OA2 having intermediates activity ($P = 0.0938$). Catalase had greater activity in the PC group than in NC pigs with both OA treatments having intermediates values ($P = 0.0364$). Pigs given 1 mL of OA/L of water (OA1) had the greater SOD activity ($P < 0.001$) than all other treatments.

The number of CFU/g for *Bifidobacterium* and *Lactobacillus* in cecal contents are shown in Table 7. No difference among treatments was detected in CFU/g for both *Bifidobacterium* and *Lactobacillus* ($P > 0.337$).

DISCUSSION

Acidifiers are often used as alternatives to antibiotic growth promoters because of their ability to modify the intestinal environment in favor of beneficial microorganisms, enhancing the intestinal health (Long et al., 2017), improving nutrient digestibility (Mao et al., 2019), increasing growth performance (Diao et al., 2016), and reducing diarrhea (Han et al, 2018). In this experiment, the addition of OA in the drinking water did not affect weaned pig growth performance. These results agree with previous studies with unprotected OA for weaned pigs (Canibe et al., 2005; Kil et al., 2005; Ahmed et al., 2014; Yang et al., 2019).

However, many other studies have found a positive effect of single [fumaric acid (Lawlor et al., 2005); K-diformate (Kluge et al., 2006); Ca-formate (Bosi et al., 2007); benzoic acid (Torrallardona et al., 2007; Diao et al., 2016; Silveira et al., 2018); citric acid (Suiryanrayna et al., 2012)] or blends of OA [butanoic, fumaric, and benzoic acid (Li et al., 2008); fumaric acid, Ca-lactate, citric acid, and medium-chain fatty acids (Kuang et al., 2015); formic, propionic, lactic and phosphoric acid (Long et al., 2017); formic, acetic, lactic, propionic, citric, and sorbic acid (Han et al., 2018)] on growth performance of weaned pigs. Inconsistency in response to feed additives for swine as an alternative to antibiotics as growth promoters common. The effects of using OA for weaned pigs may vary among different experiments due to different experimental conditions such as weaning age, diet composition, immunological challenge, the buffer capacity of the diet, and finally due to OA properties such as type, blend composition, GIT site of action, pKa, and level of inclusion. In this experiment disease incidence variability was attempted to be standardized by inoculating all pigs with *E. coli* F4 at days 3 and 4.

Most studies have been done with OA inclusion in the feed. Moreover, there is a lack of information on how OA might affect water intake. Despite physical and chemical parameters such as mineral content, hardness, total solids, and microbiological measures, the water pH might be an important driver of water consumption. In this experiment, the DWU was calculated for each experimental unit and the inclusion of OA in the drinking water reduced the pH (OA1= 3.72; OA2 = 3.28). The inclusion of OA in the drinking water increased DWU during the entire nursery phase. Consistent with our findings, Houben et al. (2015) and Escudero et al. (2016) showed that pigs had preferred lower pH water (pH=3.7), due to OA inclusion, rather than common water (pH=7.3). In the present study, OA inclusion in the drinking water significantly decreased the pH with the greatest dosage resulting in the lowest water pH. Water intake is well-known to be associated with feed intake. Biglow and Houpt (1988) described pigs as prandial drinkers, which means that about 50% of the total water intake happens during or right after meals. In this case, improvements in water consumption during the nursery phase may increase feed intake, which is crucial especially during the first week post-weaning. Additionally, the post-weaning period is characterized by the occurrence of profuse diarrhea and dehydration (Wada et al., 1996; Rhouma et al., 2017). Therefore, adequate water intake can ameliorate these clinical symptoms of weaning stress. In the present study, the improvements in DWU were not followed by an increase in feed intake. Moreover, the lack of improvements in growth performance could be explained by the

great challenge induced by the weaning dietary transition and *E. coli* challenge as well as the weaning body weight and age.

Although diarrhea incidence did not differ among treatments for each period of evaluation in this study, all treatments showed a great increase in diarrhea incidence in the second week, when compared to the first week, likely a result of the *E. coli* challenge and the dietary transition. Consistent with our results, Han et al. (2018) observed only numerically reduction of diarrhea incidence, and Xu et al. (2018) reported no difference in the fecal score comparing the use of OA and antibiotics in the diet of weaned pigs. Similarly, Yang et al. (2019) showed that protected OA (17% fumaric acid, 13% citric acid, 10% malic acid, 1.2% mix of capric and caprylic acid) were more effective in lowering diarrhea incidence and changing microbiota composition in weaned pigs than unprotected ones. In this experiment, *Lactobacillus* and *Bifidobacterium* CFU/g cecal count were not affected by the treatments, which partially explains the similar incidence of diarrhea among treatments group, and corroborates with Xu et al. (2018) findings when comparing the use of OA (benzoic acid, calcium formate, fumaric acid) and/or essential oils as substitutes of antibiotics in the diet. The reduction of luminal GIT pH, when OA are given to pigs, is one of the mechanisms of how OA improves intestinal health (Mao et al., 2019). In this study, the stomach, jejunum, and ileum pH were not affected by dietary treatments (data not shown). Xu et al. (2018) also found that OA in the diet did not influence GIT segments pH. At the moment of weaning, low gastric acid secretion, lack of lactose, the buffering capacity of the feed, and pattern of feed intake can result in elevated stomach pH. Therefore, lowering the pH with acidifiers can help pigs in the transition from milk to solid feed (Suiryanrayna and Ramana, 2015).

The incidence of belly nosing also showed an increase on the second week of evaluation when compared to the first week. Gardner et al. (2001) showed that belly nosing behavior can be an indicator of stress but is not related to diet quality, feed intake, and the presence of milk derivate ingredients in the diet. Therefore, such increase in this type of behavior could be possibly explained because of the weaning age (approximately 20 days) and body weight (5.17 kg).

Jejunum morphometry on day 9 was not affected by the inclusion of either antibiotics or OA in the diet. However, pigs that were given OA in the drinking water (OA2) had a numerical increase of 37% on villous height compared to the NC. Li et al. (2018), Long et al. (2017), Xu et al. (2018) and Manzanilla et al. (2006) also showed that OA or antibiotics were

not able to change neither jejunum nor ileum morphometry, which corroborates our findings. Intestinal injuries and an increase in gut permeability is often a potential cause for local and systemic immunological changes. Weaning is also associated with an early inflammatory response that may contribute to both anatomical and functional intestinal disorders in pigs (Pié et al., 2004). The cytokines produced during inflammation directly and indirectly influence epithelial ion transport and permeability (McKay, 1999). Thus, the high exposure to environmental and dietary antigens in addition to intestine damage may over-activate the immune system and triggers the increase of gut permeability and pathogen translocation. The decline in the number of peripheral WBC can be an indicator of monocytes/macrophages recruitment, in the early stage of infection, to the site of inflammation (Che et al. 2011). In the present study, the number of monocytes in the serum on the third day post-weaning, before *E. coli* challenge, was greater in pigs that receive OA2 in the water, indicating a beneficial delay on monocytes recruitment to sites of inflammation due to weaning stressors such as diet transition. However, no difference was found for total WBC and days of collection. These results were like those reported by Namkung et al. (2004) when feeding OA to weaned pigs and by Kil et al. (2005) when feeding different sources of acidifiers to LPS-challenge weaned pigs. On the other hand, Che et al. (2011) showed an increase in WBC when pigs were fed manno-oligosaccharides when compared to the control group. The degree of changes in WBC due to intestinal damages can be a result of multifactorial events, such as weaning age, diet composition, and pathogens load, which generates different responses to multiple feed additives approaches.

It is well recognized that weaning may disrupt all the criteria that define a healthy gut: it modifies enzyme activity (Montagne et al., 2007) and nutrient bioavailability (Suthongsa et al., 2017), impairs intestinal morphometry (Moeser et al., 2007; Suthongsa et al., 2017), modifies the normal behavior of suckling pigs (Turpin et al., 2017), super activates the immune system (Pié et al., 2004; Pluske et al., 2018), promotes modification of microbiota population (Isaacson and KIM, 2012; Bauer et al., 2006; Poulsen et al., 2018), VFA concentration in the large intestine (Franklin et al., 2002), and intestinal antioxidant capacity (Wang et al., 2017). In the present study, the absence of changes in CFU/g counts for *Lactobacillus* and *Bifidobacterium*, possibly due to pharmacological doses of zinc oxide in the diet, resulted in only small changes in VFA concentration in cecum content, with a tendency that larger dosage of OA decreases total, acetic, and propionic acids compared to the lower dosage. That can possibly be explained due to the greater amount of OA in the

cecum because of the greater dosage and water intake for this group of pigs, promoting changes in the microbiota other than the species evaluated in this study. Other feed additives have been studied as potential modulators of microbiota and consequently VFA production. Jaworski et al. (2017), evaluating direct-fed microbials (DFM) and fiber inclusion, showed that DFM did not affect VFA concentration in large intestine segments. Xu et al. (2018) also reported that OA did not change VFA concentration in the cecum and colon of weaned pigs.

Oxidative stress is a result of an imbalance of concentration ROS and levels of ROS-scavenging proteins in the serum or the tissue (Wang et al., 2017). Weaning stressors often increase oxidative stress. Antioxidant enzymes such as glutathione peroxidase, CAT, and SOD, are part of an antioxidant system that are used to protect the body from oxidative stress and cell damage (Zhan et al., 2006). Superoxide dismutase is responsible to convert superoxide radicals to non-toxic hydrogen peroxide which will be further converted to non-toxic water through CAT or glutathione peroxidase activities (Yin et al., 2014). Wang et al. (2012) studied the effects of *Lactobacillus plantarum* ZLP001 in the diet of weanling pigs when compared to antibiotics and showed that greater activities of these antioxidant enzymes can decrease the concentrations of ROS, thereby alleviating the oxidative stress. In the present study, antioxidant capacity was evaluated in the jejunum. Reduced glutathione and lipid peroxidation did not differ among treatments in the jejunum. However, greater activity of SOD in the jejunum of pigs that received OA1 in the water indicates an improvement in antioxidant capacity. Catalase activity was also increased in all treatments when compared to the negative control group. The ability of OA to ameliorate oxidative stress have been reported through improvements in serum total antioxidant capacity (Long et al., 2017), lowering lipid peroxidation (Xu et al., 2018), activities of glutathione peroxidase and SOD (Diao et al., 2016), and elimination of hydroxyl radicals (Long et al., 2017).

Most of the feed additives applied as substitutes to antibiotics can have either a direct immune modulation effect on up or down-regulating intestinal immune response or indirect contributions to the immune homeostasis, for example, promoting microbiome equilibrium. Nevertheless, the success of any strategies regarding the replacement of antibiotics as growth promoters in pig diets will depend on a combination of nutritional, management, housing, biosecurity, and health factors. It can be suggested that the microbiome equilibrium is the factor that might trigger all the other events in the gut. Thus, all strategies that are proven to promote this balance are important. In this regard, OA might be important to manipulate the

GIT microbiota to maintain animal gut health, through enhancement in diversity, stability, metabolites production, and crosstalk with the epithelium and the immune system. Yet, the use of OA should be considered as prevention of intestinal disturbances much more than a curative approach.

CONCLUSION

In summary, the OA inclusion in the drinking water of weaned pigs throughout the entire nursery phase can improve the DWU providing a better hydration status and possibly ameliorating the common consequences of diarrhea at this time. General intestinal health parameters and growth performance evaluated in this study did not differ between OA and the antibiotic colistin indicating that OA can safely replace colistin in the diet of weaned pigs with similar health improvements. In addition, the enzymatic antioxidant system in the jejunum was improved by OA in the water, which mechanistically could explain part of the positives effects of OA on weaned pigs.

In conclusion, OA constitute a sustainable and safe alternative to colistin in the diets of weaned pigs. A variety of mechanisms have been reported in the literature. Yet, synergy and antagonisms with other feed additives should be further explored.

CONFLICT OF INTEREST

No conflicts of interest, financial, or otherwise are declared by the author(s).

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Table 1. Dietary composition of basal experimental diets1 (as-fed basis).

Item	Phase I (7 d)	Phase II (7 d)	Phase III (14 d)	Phase IV (13 d)
Ingredient, %				
Corn	0.000	0.000	52.500	58.012
Micronized rice	46.500	50.500	1.938	0.000
SBM[2], 46 % CP	0.000	0.000	28.000	31.000
SBM HyPro[2], 48 % CP	14.000	16.000	2.250	0.000
Dried whey	16.800	9.600	3.125	0.000
Sugar	11.000	9.000	4.538	4.000
Ground cookies	5.237	10.199	1.809	0.000
Soybean oil,	0.000	0.000	3.000	3.000
Bovine blood plasma	2.500	1.500	0.000	0.000
Dicalcium phosphate, 19% P	0.444	0.370	0.563	0.780
Tricalcium phosphate	0.350	0.250	0.175	0.000
Salt	0.430	0.500	0.375	0.500
Hydrolyzed yeast	0.700	0.200	0.469	0.000
Nucleotides	0.050	0.042	0.000	0.000
Feed flavor and sweeteners	0.290	0.242	0.235	0.010
L-carnitine	0.010	0.008	0.000	0.000
L-Lysine sulfate, 70%	0.000	0.000	0.000	0.120
L-Lysine HCl	0.548	0.473	0.333	0.000
L-Threonine	0.288	0.242	0.068	0.004
DL-Methionine	0.253	0.199	0.076	0.004
Phosphatidylcholine	0.000	0.000	0.000	0.008
BHT	0.013	0.009	0.005	0.010
Enzymes	0.010	0.010	0.010	0.008
Phytase 10000 FTU	0.010	0.008	0.010	0.010
Calcium carbonate	0.000	0.200	0.188	0.848
Copper sulfate	0.019	0.016	0.016	0.014
Tribasic copper chloride	0.012	0.010	0.010	0.012
Mineral premix[3]	0.080	0.070	0.080	0.080

Vitamin premix[4]	0.125	0.113	0.092	0.015
Kaolin	0.000	0.000	0.000	1.429
Zinc oxide, 80%	0.331	0.239	0.138	0.138
Calculated values				
ME, kcal/kg	3552.0	3559.0	3515.0	3403.0
Crude protein, %	15.600	15.690	19.700	19.430
Calcium, %	0.541	0.511	0.574	0.790
Phosphorus, %	0.637	0.560	0.635	0.630
Available phosphorus, %	0.473	0.381	0.420	0.422
SID[5] Lysine, %	1.330	1.230	1.302	1.110
SID Methionine, %	0.536	0.484	0.432	0.361
SID Threonine, %	0.921	0.861	0.822	0.750
SID Tryptophan, %	0.214	0.211	0.260	0.230
Sodium, %	0.419	0.372	0.213	0.216
Zinc, mg/kg	2750	1948	1700	1700

[1]For the positive control diet phase I included 150 ppm of colistin sulfate, phase II 120 ppm of colistin sulfate, and phases III and IV 80 ppm of colistin sulfate, at the expense of rice or corn in the basal diet.

[2]SBM: soybean meal, 46% crude protein; SBM HyPro: soybean meal 48 % crude protein.

[3]Levels per kg of diet, mineral premix: 80.0 mg of iron, 35.0 mg of manganese, 0.45 mg of selenium, and 1.0 mg of iodine.

[4]Levels per kg of diet, vitamin premix: 13,000 IU of vitamin A, 2,250 IU of vitamin D3, 100.0 IU of vitamin E, 4.0 mg of vitamin K3, 3.5 mg of vitamin B1, 8.0 mg of vitamin B2, 20 mg of pantothenic acid, 6.0 mg of vitamin B6, 35.0 mcg of vitamin B12, 50 mg of nicotinic acid, 0.35 mg of folic acid, and 0.30 mg of biotin.

[5]Standardized ileal-digestible

Table 2 - Effect of different organic acids dosage in the drinking water on nursery pigs (n=112) growth performance and daily water usage (DWU).

Item	Treatments[1]				SEM	*P*-Value
	NC	PC	OA1	OA2		
Initial BW	5.17	5.17	5.17	5.17	0.18	1.0000
0 to 7 days						
d 7 BW, kg	5.31	5.38	5.31	5.29	0.21	0.9923
ADG, g	19.39	29.59	20.51	17.91	14.19	0.9366
ADFI, g	76.51	82.03	84.82	74.97	10.69	0.9043
Gain:feed	0.28	0.39	0.21	0.33	0.13	0.7711
DWU, L	1.03^{b}	1.28^{ab}	1.43^{ab}	1.80^{a}	0.18	0.0445
8 to 14 days						
d 14 BW, kg	6.33	6.30	6.27	6.16	0.25	0.9698
ADG, g	145.78	131.59	136.71	124.17	12.87	0.6875
ADFI, g	315.63	272.03	279.72	285.01	14.63	0.1619
Gain:feed	0.46	0.49	0.49	0.44	0.05	0.8312
DWU, L	1.24^{b}	1.44^{b}	1.82^{b}	3.00^{a}	0.21	<0.0001
15 to 21 days						
d 21 BW, kg	8.25	8.30	8.16	7.74	0.31	0.5630
ADG, g	275.55	285.97	270.23	225.37	49.07	0.8271
ADFI, g	310.12	341.17	351.29	302.90	18.84	0.1984
Gain:feed	0.85	0.84	0.67	0.74	0.17	0.5762
DWU, L	1.88^{b}	2.42^{b}	2.39^{b}	3.81^{a}	0.21	<0.0001
22 to 41 days						
d 41 BW, kg	16.81	17.96	17.48	17.21	0.81	0.7828
ADG, g	427.74	483.19	465.90	473.62	38.42	0.7542
ADFI, g	736.63	830.78	786.11	759.43	39.17	0.3823
Gain:feed	0.57	0.58	0.60	0.58	0.03	0.9556
DWU, L	2.17^{b}	2.65^{ab}	2.66^{ab}	2.93^{a}	0.15	0.0106
0 to 41 days						
ADG, g	283.90	312.04	300.25	293.77	17.47	0.7150
ADFI, g	479.23	523.96	528.46	483.68	21.52	0.2136
Gain:feed	0.59	0.59	0.61	0.60	0.03	0.7806
DWU, L	1.78^{b}	2.19^{ab}	2.26^{ab}	2.79^{a}	0.14	0.0005

^{a,b}Within a row, means with different letters differ by Tukey test ($P < 0.05$).

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

Table 3 – Effect of different organic acids dosage on the drinking water pH.

Period	Treatments[1]				SEM	*P*-Value
	NC	PC	OA1	OA2		
Week 1	6.92[a]	6.86[a]	3.76[b]	3.39[c]	0.063	<0.0001
Week 2	7.23[a]	7.20[a]	3.80[b]	3.41[c]	0.050	<0.0001
Week 3	7.27[a]	7.22[a]	3.67[b]	3.42[c]	0.067	<0.0001
Week 4	7.27[a]	7.22[a]	3.70[b]	3.38[c]	0.067	<0.0001
Week 5	6.82[a]	6.75[a]	3.77[b]	3.13[c]	0.071	<0.0001
Week 6	6.70[a]	6.63[a]	3.59[b]	2.95[c]	0.071	<0.0001
Overall	7.04[a]	6.98[a]	3.72[b]	3.28[c]	0.049	<0.0001

[a,b,c]Within a row, means with different letters differ by Tukey test ($P < 0.05$).

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

Table 4 - Effect of different organic acids (OA) dosage in the drinking water on nursery pigs (n=28) immune white blood cells (WBC).

WBC	Treatments[1]				SEM	*P*-Value
	NC	PC	OA1	OA2		
Day 1						
Leucocytes, 1000 cells/mm^3	18.9	18.5	23.0	31.1	10.68	0.606
Neutrophils, %	66.4	68.0	72.7	70.3	0.37	0.498
Lymphocytes, %	29.2	26.4	23.5	24.2	0.12	0.899
Monocytes, %	2.00	2.14	1.14	1.72	0.45	0.507
Day 3						
Leucocytes, 1000 cells/mm^3	11.6	13.3	14.7	14.3	1.49	0.493
Neutrophils, %	39.6	44.4	45.9	49.4	4.47	0.488
Lymphocytes, %	55.9	51.0	49.6	44.4	4.60	0.392
Monocytes, %	3.3^b	3.2^b	4.4^b	9.4^a	2.42	0.022
Day 7						
Leucocytes, 1000 cells/mm^3	14.4	16.5	13.4	10.0	9.80	0.821
Neutrophils, %	44.4	57.0	46.9	49.3	4.76	0.295
Lymphocytes, %	51.3	38.3	48.0	43.7	4.46	0.220
Monocytes, %	1.6	1.9	1.6	2.9	0.47	0.198

[a,b]Within a row, means with different letters differ by Tukey test ($P < 0.05$).

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

Table 5 - Effect of different organic acids dosage in the drinking water on nursery pigs (n=28) intestinal villous height, crypt depth, and villous:crypt ratio on day 9 postweaning.

Morphometry (µM)	**Treatments[1]**				**SEM**	***P*-Value**
	NC	PC	OA1	OA2		
Villous height (V)	271.5	328.2	303.3	372.0	33.68	0.2189
Crypt depth (C)	212.6	226.8	208.8	223.9	11.97	0.6682
V:C ratio	1.33	1.53	1.61	1.74	0.209	0.5857

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

Table 6 - Effect of different organic acids dosage in the drinking water on nursery pigs (n=28) redox parameters in jejunal tissue harvested on day 9 postweaning.

Redox parameters[2]	Treatments[1]				SEM	*P*-Value
	NC	PC	OA1	OA2		
GSH, µg /mg tissue	615.29	522.02	482.60	583.64	165.410	0.8527
GST, mmol/min/mg prt	100.07^{xy}	75.06^{y}	120.67^{x}	92.05^{xy}	12.690	0.0938
CAT, nmol/min/mg prt	74.49^{b}	132.50^{a}	104.41^{ab}	113.91^{ab}	13.582	0.0364
SOD, U/mg prt	830.68^{b}	685.77^{b}	1705.17^{a}	938.64^{b}	124.021	<0.0001
LPO, nmol/min/mg prt	1.56	1.18	2.03	1.45	0.335	0.2894

^{a,b}Within a row, means with different letters differ by Tukey test ($P < 0.05$).

^{x,y}Within a row, means with different letters tended to be different by Tukey test ($0.05 < P < 0.10$)

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

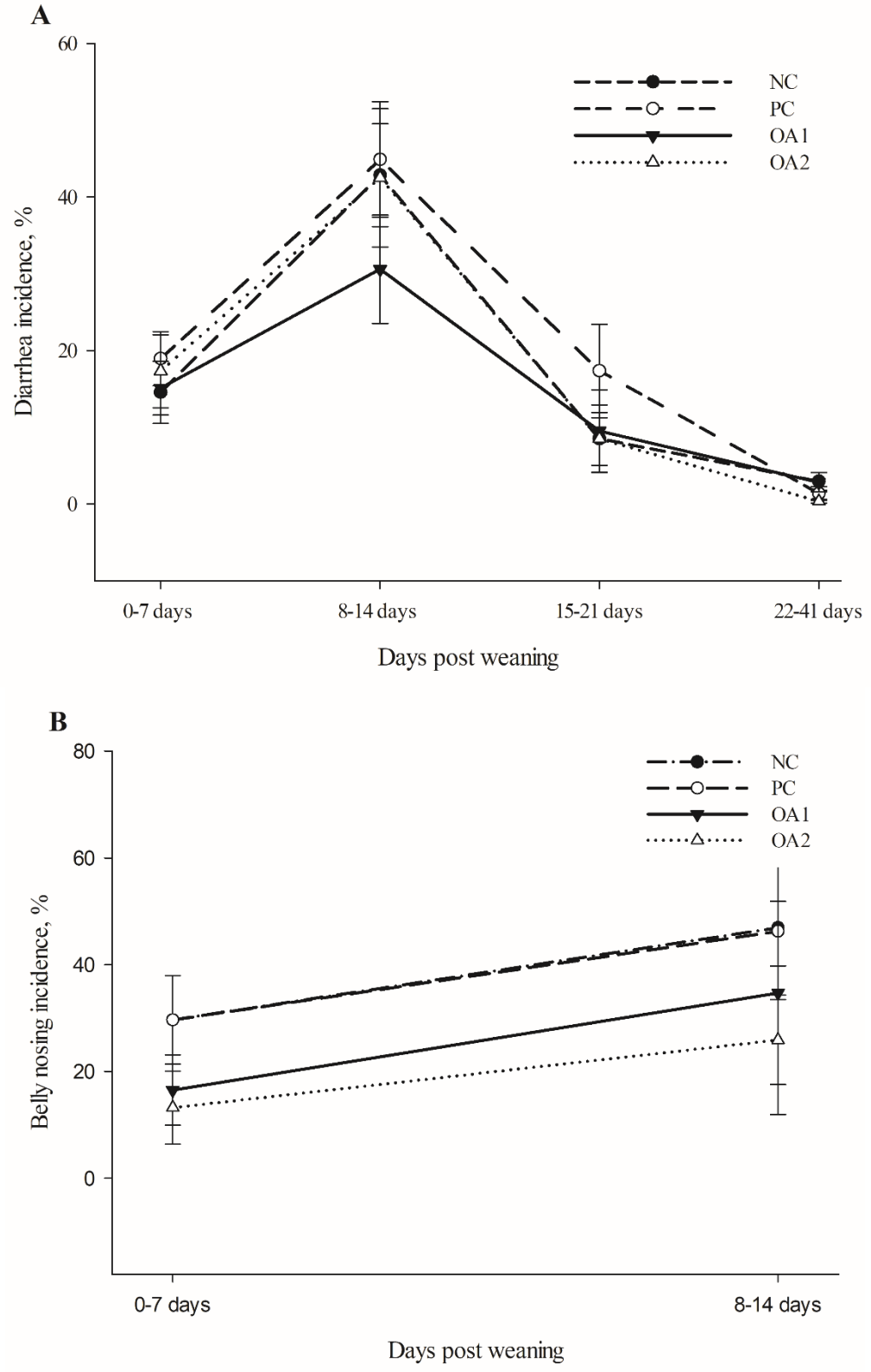
[2]GSH (Glutathione), GST (glutathione-S-transferases), CAT (Catalase), SOD (Superoxide dismutase), LPO (Lipid peroxidation) and prt (protein).

Table 7 - Effect of different organic acids dosage in the drinking water on nursery pigs (n=28) CFU/g for *Bifidobacterium* and *Lactobacillus* in cecal contents harvested on day 9 postweaning.

CFU/g Log_{10}	**Treatments[1]**				**SEM**	***P*-Value**
	NC	PC	OA1	OA2		
Bifidobacterium	7.94	7.97	8.92	7.65	0.531	0.3371
Lactobacillus	8.69	8.45	8.48	8.68	0. 416	0.9458

[1]NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

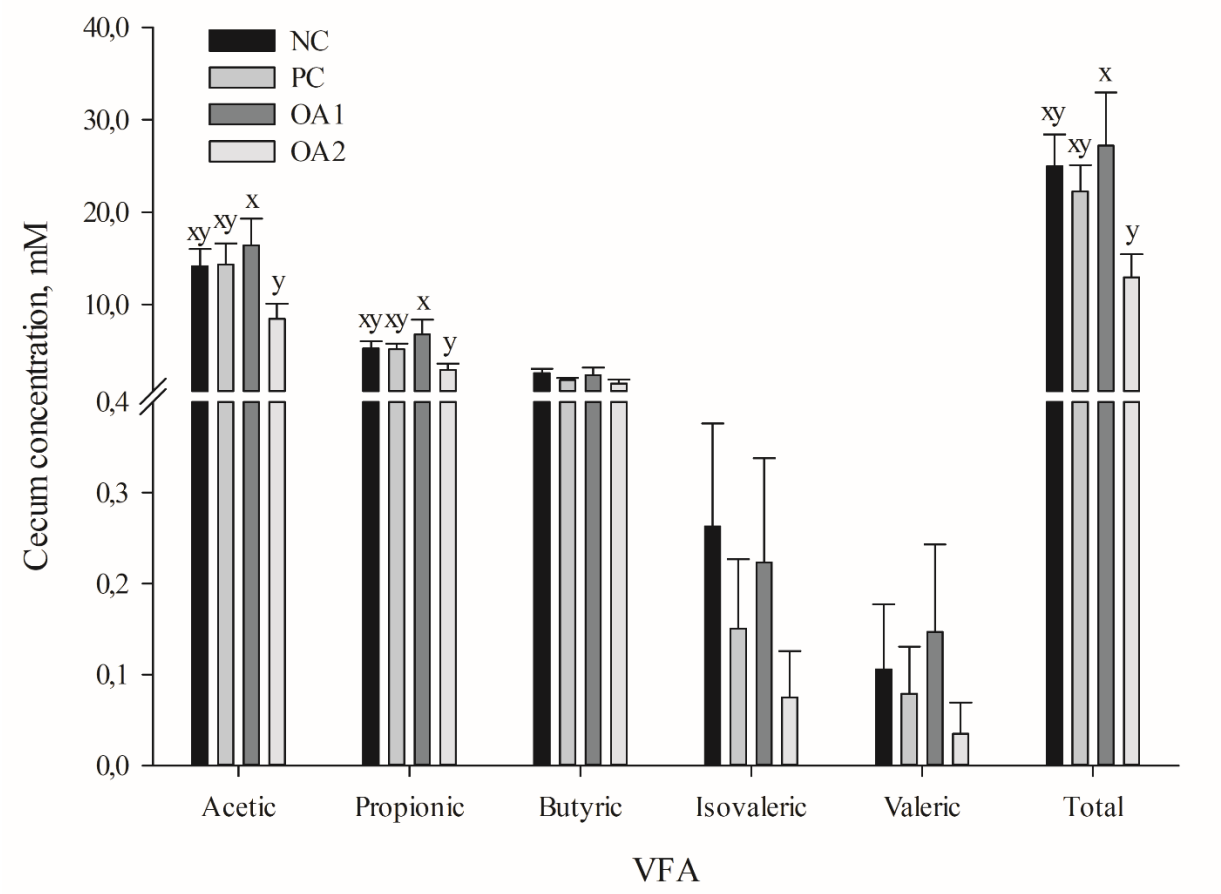
Figure 1 - Effect of different organic acids dosage in the drinking water on nursery pigs diarrhea **(A)** and belly nosing incidence **(B)**.



NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water);

OA2.0: (NC + 2.0 mL/L of organic acid blend in the water). Standard errors for the means are indicated by the vertical bars. Diarrhea incidence was calculated as the percentage of the total number of observations per pen over the period. Means were calculated for each period matching the pig growth performance periods. Belly nosing behavior was recorded when pigs presented repeated rhythmic up-and-down massage with the snout on another pig midsection (Fraser, 1978). Belly nosing behavior incidence was calculated as the percentage of the total number of observations per pen over the period. Means were calculated for first and second weeks postweaning.

Figure 2 - Effect of different organic acids dosage in the drinking water on nursery pigs volatile fatty acids (VFA) concentration in cecum contents (mM) on day 9 postweaning.



Data are shown as least square means (n = 28). [x,y]Means with different letters tended to be different by Tukey test ($0.05 < P < 0.10$). Standard errors for the means are indicated by the vertical bars. NC: Negative Control; PC: Positive Control (NC + 150; 120; 80; and 80 mg/kg of colistin sulphate in each phase step-down); OA1: (NC + 1.0 mL/L of organic acid blend in the water); OA2.0: (NC + 2.0 mL/L of organic acid blend in the water).

CHAPTER 3

Article - Effect of dacitic tuff breccia in nursery diets on pig growth performance, blood parameters, intestinal histomorphology, and nutrient digestibility

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Feed ingredients for weaned pigs

Effect of dacitic tuff breccia in nursery diets on pig growth performance, blood parameters, intestinal histomorphology, and nutrient digestibility[1]

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ABSTRACT: Two experiments were conducted to evaluate the effect of a dacitic tuff breccia (DTB), classified as a hydrated sodium calcium aluminosilicate, in the diet of nursery pigs. Experiment 1 dietary treatments were: 1) Control diet (CON), 2) CON + 0.25% DTB (DTB1), and 3) CON + 0.50% DTB (DTB2). Pigs (6.8 ±0.06 kg; 28 ±0.5 d of age, barrows) were individually housed and fed dietary treatments for 21 d (n=48). At the end of the experiment, blood, jejunal, and ileal samples were collected for a blood metabolic panel and

intestinal histologic and morphologic measurements. In Exp. 2, treatments were: 1) Control (CON) and 2) CON+0.50% DTB (DTB). Pigs (7.73 ±0.21 kg; 26 ±0.2 d of age, barrows) were individually housed and fed for 20 d (n=24). Nutrient digestibility was evaluated using individual metabolism pens for 3 d total collection of feces and urine during the last week of evaluation. In Exp. 1, DTB2 fed pigs tended to have greater ADG (P=0.07) than CON and DTB1 fed pigs, and greater ADFI (P=0.008). Gain:Feed did not differ (P=0.75) among treatments. Crypt depth in the jejunum was greater (P=0.04) for DTB1 compared to CON fed pigs but did not differ from DTB2 fed pigs. Villous height, villous:crypt, number of Goblet cells and intraepithelial lymphocytes were not different among treatments in both ileum and jejunum (P>0.17). Hematological parameters, serum proteins, serum minerals, and total white blood cell (WBC) count did not differ (P>0.10) among treatments. However, the proportion of WBC that were monocytes was lower (P=0.024) in DTB1 compared to CON and DTB2 fed pigs. Blood urea nitrogen (BUN) concentration tended (P=0.06) to be linearly decreased with increasing DTB concentrations. In Exp. 2, DTB had no effect on pig performance during week 1 (P>0.28). Week 2 ADG (P=0.02), BW (P=0.03), and G:F (P=0.02) were improved for DTB compared to CON fed pigs. Week 3 ADFI was greater for DTB fed pigs compared to CON (P=0.01). Overall (d 0-20), DTB fed pigs had greater ADG (P=0.04), final BW (P=0.04), and numerically greater ADFI (P=0.16) and G:F (P=0.22) compared to CON. Overall nutrient digestibility was not affected by inclusion of DTB in the diet (P>0.05). In conclusion, DTB inclusion in nursery pig diets improved growth performance, increased jejunal crypt depth, and tended to decreased BUN linearly and improve nitrogen digestibility. The addition of DTB in nursery diets can maximize growth performance of nursery pigs.

Keywords: hydrated sodium calcium aluminosilicate; mycotoxins; nitrogen balance; nutrition.

List of abbreviations: AFLA, aflatoxins; DTB, dacitic tuff breccia; DON, Deoxynivalenol; GIT, gastrointestinal tract; HSCA, hydrated sodium calcium aluminosilicate; WBC, white blood cells; ZEA, zearalenone.

INTRODUCTION

Several feed additives have been used to improve growth performance and health of nursery pigs (Liu et al., 2018) as the need for sustainable pork production has increasingly become a concern globally. Sustainability in swine production can be achieve through

improvements in feed efficiency, enhancements in nutrient digestibility, lowering mineral excretions, and ensuring health and welfare to animals. Nevertheless, pigs are constantly being challenged by emergent diseases, environmental problems such as heat stress, and feedstuff quality and contaminants.

Mycotoxins have important negative effects in all stages of swine life cycle. For instance, impacts of zearalenone (ZEA) on reproduction are characterized by poor oocyte quality (Xu et al., 2020), decreased number of pigs born (Zhang et al., 2015), prolonged wean to estrus interval, and increased embryonic mortality rate (Young et al., 1990). Deoxynivalenol (DON; Holanda and Kim, 2020) and aflatoxins (AFLA; Harper et al., 2010) often negatively affect growth performance and liver function in young pigs. In general, mycotoxins may impair the reproduction, hepatic, intestinal, and immunological functions in farm animals (Lindeman et al., 1993). Mycotoxin binders are important feed additives in swine diets as some of their negative effects are often subclinical and can be mitigated with compounds such as hydrated sodium calcium aluminosilicate (HSCA; Xu et al., 2020; Harper et al., 2010).

Trace minerals have several functions in organisms. Differently than macro minerals included in the diets, trace minerals are not part of tissue or cells constituents, except for hemoglobin (iron) and thyroxin (iodine). However, their role is associated with enzymatic catalytic processes and the symptoms of their deficiencies are well described in the literature (Perez, 1978; NRC, 2012). Other than their metabolic functions, several attributes of trace minerals have been studied. Over the last decade, trace minerals such as zinc and copper in pharmacological concentrations (Shannon and Hill, 2020), and selenium (Se; Doan et al., 2020) have been used for their specific functions on controlling gut pathogenic bacteria and improvements in antioxidant capacity, respectively. Yet, the roles of other trace minerals on performance of pigs remains not fully understood. The objective of this experiment was to evaluate the effects of dacitic tuff breccia (DTB; Azomite®), a feed additive classified as HSCA that contains trace minerals and rare earth elements, in the diet of nursery pigs.

MATERIALS E METHODS

General

All experimental procedures and animal housing were reviewed and approved by the Purdue University Animal Care and Use Committee (protocol #1303000841). In general,

animal care followed the Guide for Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Sciences Societies, 2010) and individual pig therapeutic medication treatments were given and recorded when pigs exhibited clinical signs of illness following the Purdue Veterinary Care SOP. The experiment was performed in the Animal Sciences Research and Education Center (ASREC) at Purdue University, West Lafayette, IN 47906, U.S.A. Pigs were weaned at approximately 20 d of age and were fed a common pelleted starter diet for 7 d postweaning in the pre-study period to assure pigs were eating well and healthy before being moved to individual pens. Pigs were individually housed in fully slatted plastic floor pens (0.41 x 0.86 m; 0.34 m^2/pig) with a single nipple waterer and a 2-hole feeder in front of the pen. The growth performance was determined by weighing pigs and feed disappearance at beginning (d 0) and end (d 21) for Exp. 1 and weekly for Exp. 2. The dacitic tuff breccia (DTB, Azomite®) product included in the diet was provided by Azomite (Nephi, UT). The product is classified as HSCA, listed as a feed additive for livestock animals and considered safe by the United States Food and Drug Administration (FDA). All diets in these experiments contained antibiotics or antimicrobials but did not contain pharmacological levels of zinc or copper. The mineral and rare earth elements composition of the DTB is shown in Table 1. Diets were analyzed for major mycotoxins levels for both trials (Table 2, Exp. 1; Table 3, Exp. 2).

Experiment 1

A total of 48 crossbred barrows [6.8 +0.06 kg; Duroc x (Landrace x Yorkshire)], 28 ±0.5 d of age, were individually weighed, blocked by body weight, and randomly allotted to 1 of 3 treatments with 16 replicates: **CON** (control diet), **DTB1** (CON + 0.25% DTB), and **DTB2** (CON + 0.50% DTB). Pigs were housed in individual pens and allowed *ad libitum* access to water and feed over 21 d. Dietary treatments were fed in two phases, phase for 7 d and phase 2 for 14d (Table 4). The corn-soybean meal-based diets were in meal form and formulated to meet or exceed nutrients requirements (NRC, 2012). A mixture of the test article and finely ground corn replaced corn in the experimental CON diet at the planned rate of concentration in feed to create experimental treatment DTB1 and DTB2.

Blood samples (n = 24) were collected from the jugular vein at the end of the study (d 21) in EDTA tubes (2 mL/pig) for blood chemistry and hematology panel analysis at Purdue University College of Veterinary Medicine Clinical Pathology Laboratory (West Lafayette, IN) using the Siemens Advia 2120i system for hematology (Siemens Healthcare Diagnostics

Inc; Malvern, PA U.S.A) and Ortho Diagnostics Vitros Fusion 5.1 for blood chemistry (Ortho Clinical Diagnostics Inc; Rochester, NY, U.S.A). The metabolic parameters assayed included glucose, creatinine, and blood urea nitrogen (BUN). The biochemistry analysis included: serum total protein, plasma total protein, albumin, globulin, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transferase. The minerals concentration analysis included: calcium, sodium, potassium, phosphorus, magnesium, and chloride. The hematology analysis included: hematocrit, hemoglobin, red blood cells count, white blood cells (WBC) count, lymphocytes, monocytes, and neutrophils as a percent of WBC.

The same pigs that had blood collected (n = 24) were euthanized using a carbon dioxide chamber followed by exsanguination for tissue harvest. Jejunal (2.0 m posterior from pylorus) and ileal (0.5 m from the ileocecal junction) cross sections were collected, rinsed with phosphate buffer solution, and preserved in 10% neutral buffered formalin. Intestine samples were cut in a microtome (5 μm thickness), and stained in hematoxylin and eosin for intestinal morphometry, in Alcian blue for Goblet cells count, and immunohistochemical staining for CD3 protein detection for intraepithelial lymphocytes count (Fig. 1, Fig. 2). Six well oriented villous and crypts were analyzed using the Leica Aperio system (Buffalo Grove, IL, U.S.A), for evaluation of villous height and crypt depth. The same six villous and crypts used for intestinal morphometry were used for goblet cells and intraepithelial lymphocytes count. The total number of columnar cells, positively stained Globet cells, and positively stained intraepithelial lymphocytes were counted for each pig sample (n=24). The proportion of Goblet cells and intraepithelial lymphocytes were calculated as number of cells per 100 columnar cells.

Experiment 2

A total of 24 crossbred barrows [7.7 ±0.21 kg; Duroc x (Landrace x Yorkshire)], 26 ±0.2 d of age, were individually weighed, blocked by body weight, and randomly allotted to 1 of 2 treatments: **CON** (control diet) and **DTB** (CON + 0.50% DTB). Pigs were allowed *ad libitum* access to water and feed via a single nipple drinker and single feeder over 20 d in two dietary phases, phase 1 for 7 d and phase 3 for 13 d (Table 5). The corn-soybean meal-based diets in meal form were formulated to meet or exceed nutrients requirements (NRC, 2012). A mixture of the test article and finely ground corn replaced corn in the experimental CON diet at the planned rate of concentration in the feed to create experimental treatment DTB. The phase 2 diet contained chromic oxide, added as an indigestible marker in the diets. The first

14 days were used as an acclimation phase to the individual pens (0.41 x 0.86 m; 0.34 m^2/pig) to avoid confounding factors caused by the stress of this type of housing. Then, the nutrient digestibility study took place the last seven days. During the collection phase, pigs were transferred and individually housed in metabolism pens (0.63 m^2/pig) with a single nipple waterer and a 1-hole feeder in front of the pen.

Nutrient digestibility was evaluated using individual metabolism pens for 3 d total collection of feces, urine, and orts. Feces were collected on screens with a separate collection of spilled feed and water from the front of the crate. Urine was collected in pans to a plastic bucket containing 50 mL of 10% hydrochloric acid under each pen with a separate collection of spilled water from the front of the crate. The urine collected was measured and 10% subsample was retained daily. Feces and urine subsamples were labeled and stored at -20°C prior to preparation for analysis. During the collection phase, the feed allowance was three times the maintenance energy requirements according to NRC (2012) and their respective BW upon entry of the phase. The total daily feed was divided into 2 meals and given to pigs at 0800 and 1500 h and feed refusal or orts was carefully recorded daily.

Feces collected were thawed and then homogenized with a known quantity of water and a subsample was freeze dried. Urine subsamples were thawed prior to assays. Experimental diets were finely ground using a centrifugal grinder (ZM200; Retsch GmbH, Haan, Germany). All samples were analyzed for DM [procedure 934.01, Association of official Analytical Chenmists (**AOAC**), 2006], ash (procedure 942.05, AOAC, 2006), Ca (procedure 968.08, AOAC, 2006), and N by LECO combustion (procedure 990.03, AOAC, 2006). Feces, urine, and experimental diets were analyzed for gross energy (GE) using an isoperibol bomb calorimeter (Parr 6200; Parr Instrument Co., Moline, IL). For urine energy, 3 mL of urine was dried for 24 h on approximately 2 g of cellulose prior to combustion. Diets and feces samples were digested in perchloric acid (procedure 965.17, AOAC, 2006) for determination of P using UV-visible spectrophotometer (TECAN, Model Spark 10M) using a wavelength of 650 nm (procedure 965.17, AOAC, 2006). Sodium, Cr, Mg, Zn, Fe, and Mn were analyzed in all samples using flame atomic absorption spectrophotometer (Varian, Model SpectrAA 220FS) using the manuals specified wave length and lamp for each mineral (procedure 968.08, AOAC, 2006). Diets were also analyzed for ADF, NDF, and AA.

The N digestibility (**Nd**) was calculated using:

$$Nd = [(Ni – Nf) /Ni] \times 100,$$

where Nd is N igestibility (%), Ni is N intake (g), and Nf is fecal N output (g).

The apparent total tract tract digestibility (**ATTD**) for gross energy was calculated using:

$$\text{ATTD of GE} = [(GEi – GEf) / GEi] \times 100,$$

where ATTD of GE is the gross energy digestibility (%), GEi is GE intake (kcal/kg), and GEf is fecal GE output (kcal/kg).

The metabolizability of gross energy was calculated using Eq. [x]:

$$\text{metabolizability of GE} = [(GEi – GEf - GEu) / GEi] \times 100,$$

where GEi is GE intake (kcal/kg), GEf is fecal GE output (kcal/kg), and GEu is urinary GE output (kcal/kg). Digestible energy (**DE**) of the diets were obtained by multiplying the GE (kcal/kg) in the diet by ATTD of GE and metabolizable energy (**ME**) by multiplying the GE (kcal/kg) in the diet by metabolizability of GE.

The ATTD for, P, Ca, Na, Zn, Fe, and Mn was calculated using Eq. [x]:

$$\text{ATTD of mineral} = [(Mi – Mf)/Mi] \times 100,$$

where is Mi is mineral intake (g), and Mf is fecal mineral output (g).

Statistical analysis

All variables were tested for normality using Shapiro-Wilk test and those that failed to follow a normal distribution were transformed through the RANK procedure from SAS (SAS Inst. Inc., Cary, NC). All data were analyzed using the PROC MIXED procedure from SAS (SAS Institute Inc., Cary, NC, U.S.A) as a randomized complete block design (initial BW). The pig was the experimental unit for all calculations. All data are reported as least squares means, compared by Tukey test, and standard errors for the means are reported. Means were considered significant different when $P \leq 0.05$ and trends were discussed when $0.05 < P \leq 0.10$. The frequency of medication therapies, as binary distributions, were analyzed using PROC FREQ procedure from SAS (SAS Institute Inc., Cary, NC, U.S.A) and chi-square probabilities were reported.

RESULTS

Growth performance

Growth performance results are shown in Table 6 (Exp. 1) and Table 7 (Exp.2). In Exp. 1, DTB2 fed pigs tended to have greater ADG (P = 0.0679) than CON and DTB1 fed pigs resulting in greater BW gain (P = 0.0665) and numerically 5% heavier pigs at the end of the study (P = 0.1092). DTB2 fed pigs had greater (P=0.0083) ADFI throughout the experimental period than CON and DTB1 pigs. Gain:Feed was not affected (P = 0.7512) by dietary treatments. In Exp. 2, DTB in the diet did not affect pig performance during in the first week of study (P > 0.26). In the second week, ADG (P = 0.0151), BW (P = 0.0335), and G:F (P = 0.0178) were improved and ADFI was numerically greater (P = 0.1378) for DTB compared to CON pigs. In the third week, ADFI was greater for DTB fed pigs compared to CON (P = 0.0144) while ADG (P = 0.1641) and G:F (P = 0.3274) did not differ. During the 3 d collection phase in metabolism crates during week 3, DTB fed pigs had greater ADFI (P = 0.0262) whereas ADG and G:F did not differ (P > 0.32). For the overall experimental period (d 0-20), DTB fed pigs had greater ADG (P = 0.0421), final BW (P = 0.0388), and numerically greater ADFI (P = 0.1603) and G:F (P = 0.2245) compared to CON.

Blood metabolic parameters

The blood metabolic parameters results from Exp.1 are shown in Table 8. The inclusion of DTB in the diet numerically decreases the urea concentration in the serum (P= 0.1318) and did not affect the glucose level (P = 0.7777). No effects were observed on serum (P = 0.3891) nor plasma (P = 0.5028) total protein concentration as well as albumin (P = 0.2937), globulin (P = 0.4384), and hepatic enzymes (P > 0.15). None of the mineral concentrations analyzed in the serum were affected by dietary treatments (P > 0.32). The number of red cells (P = 0.1243), the hemoglobin concentration (P = 0.6747), and the hematocrit ratio (P = 0.5199) did not differ among dietary treatments. The overall WBC count was not affected by DTB inclusion in the diet. However, the proportion of monocytes was lower for DTB1 fed pigs than CON and DTB2 (P = 0.0236).

Intestinal histomorphology

Intestinal histomorphology, number of Goblet cells, and intraepithelial lymphocytes in the jejunum and ileum are shown in Table 9. The villous height in the jejunum (P = 0.8772) and in the ileum (P = 0.7134) were not influenced by DTB inclusion in the diet. The crypt depth in the ileum (P = 0.8483) did not differ among treatments but were deeper in the

jejunum of pigs fed DTB1 than CON and the DTB2 jejunal crypts were intermediate to both treatments (P = 0.0401). The villous:crypt ratio was not different in jejunum nor ileum (P > 0.81) among treatments. The number of intraepithelial lymphocytes in the ileum linear decreased numerically (P = 0.2373) while goblet cells linear increased numerically (P = 0.1678) with DTB increasing concentration and were not affected in the jejunum (P > 0.60).

Nutrient digestibility

Pigs did not show any sign of feed refusal or difficult eating the DTB diet and no level above limits of mycotoxin were detected in the diets for both trials. The feed allowance during the collection phase was three times the maintenance energy requirements according to NRC (2012) and their respective BW upon entry the phase. Dry matter digestibility, energy and N balance are shown in Table 10.

The CON fed pig's entry BW was 11.10 kg and DTB fed pigs was 12.47 kg (P= 0.0218) which resulted in greater feed allowance and ADFI for DTB pigs than CON (544 vs 668 g, P = 0.0262). The total gross energy intake was greater for DTB fed pigs than CON (P = 0.0457) but did not differ in the feces (P = 0.1430) nor in the urine (P = 0.8504) energy. The energy gross digestibility (P = 0.9647) and metabolizability (P = 0.9739) did not differ between dietary treatments.

Pigs fed DTB had greater N intake than CON (P = 0.0287) but no difference was found in the N excretion in the feces (P = 0.6749). The grams of N absorbed tended to be greater in DTB fed pigs than CON (P = 0.0627) whereas N digestibility was numerically 12% greater for pigs fed DTB than CON (P = 0.1268).

Minerals digestibility are shown in Table 11. Phosphorus, calcium, sodium, zinc, iron, and manganese digestibility did not differ between treatments (P > 0.05).

Therapeutic medication treatments

The frequencies of medication therapies for signs of diarrhea for Exp.1 and Exp.2 are shown in Table 12. No differences on the number of therapies were observed among treatments in Exp.1. In Exp 2, there was no difference between treatments for the frequency of therapies (P>0.05) but pigs fed DTB had a numerical lower need of a second drug medication therapy than CON (P=0. 2191).

DISCUSSION

Trace minerals have several functions on physiology, metabolism, and health of animals. Trace mineral supplementation can have an important impact on growth and on the maintenance of well-balanced microbiota of pigs (Shannon and Hill, 2020) whereas the functions and needs of rare earth elements remain unclear. The use of HSCA have been studied in different pig ages and stages to ameliorate the impacts of mycotoxins in swine diets.

Studies feeding DTB to other farm animals as a feed additive have been reported. The growth performance of chicks (d 0-21; Jones et al., 2018), broilers (Pirzado et al., 2020a), and the feed conversion of hens (Malheiros et al., 2018) were improved with the inclusion of DTB in the diet. The addition of DTB in the feed of aquatic species have been studied over the last decade. The growth performance, immune function, and digestives enzymes activities were improved in white shrimp (*Litopenaeus vannamei*; Tan et al., 2014), in largemouth bass (*Micropterus salmoides*; Xu et al., 2021), in koi carp fingerlings (*Cyprinus carpio*; Jaleel et al., 2015), in catfish (*Pangasius hypophthalmus*; Batool et al., 2018), and in grass carp (*Ctenopharyngodon idella*; Liu et al., 2011) fed DTB. In tilapias (*Oreochromis niloticus* × *O. aureus*), growth performance, immune function, nutrient digestibility, and intestinal morphometry were improved with DTB in the diet (Liu et al., 2009). Moreover, Azam et al. (2016) showed that the growth performance, digestives enzymes activities, and body composition of male tilapias (*Oreochromis niloticus)* were improved with DTB in the diet. In addition, in male tilapias fed DTB (*Oreochromis mossambicus),* milt quality and gonadal hormones levels were improved (Ahamed et al., 2020).

In the present study, like other species, young pigs had their growth performance improved when fed DTB during the nursery phase. A limited number of studies were conducted to evaluate DTB for pigs. Improvements in growth performance of young pigs, in early stages of the nursery phase, were observed by Richert et al. (2021) through greater ADG and ADFI in phase 2 (27 -34 d old) and better feed efficiency at phase 3 (35 – 42 d old) for pigs fed DTB than a negative control, which corroborates the results of the present study. On the other hand, Chevalier eta. (2021) showed no effects on growth performance of growing pigs (BW 36 – 61 kg) individually housed and fed DTB for 28 d.

Mycotoxins in animal feed is a worldwide concern. Corn is the major grain used in swine diets, is commonly contaminated with these secondary fungal metabolites which causes toxicity and growth retardation in pigs (Harper et al., 2010). Harvey et al. (1989) have

first studied the effects of HSCA and compared different sources of HSCA (Harvey et al., 1994) on amelioration of induced-aflatoxicosis in growing pigs showing improvements in growth performance and clinical signs of aflatoxicosis. Moreover, the negative impacts of ZEA on pig oocytes quality were reduced by the inclusion of HSCA in ZEA-contaminated diets fed to sows (Xu et al., 2020). On the other hand, no effects of HSCA on growth performance of pigs fed DON contaminated diets (Patterson and Young, 1993) neither on growth nor carcass traits of finishing pigs (Matthews et al., 1999) were observed. However, HSCA improved growth performance of young pigs fed aflatoxin moderate-contaminated diets (Harper et al., 2010). In this study, corn used in the diet had no known levels of AFLA and DON at the time of harvest which was common according to the region (Central Indiana, U.S.A) as well as no level above limit for the major mycotoxin in the diets as fed. No clinical signs of mycotoxicosis were observed in both trials. In an *in vitro* study, the adsorption affinity is greater for aflatoxins and fumonisin whereas the binding affinity for DON appeared to be non-specific binding (Tejeda et al., 2019). Therefore, the ADFI and ADG improved in DTB fed pigs, are unlikely to be related to subclinical signs of mycotoxins in young pig diets.

Metabolic parameters including mineral concentrations, biochemistry, and hematological analysis, except for BUN and proportion of monocytes, did not differ among dietary treatments. Nevertheless, all parameters were within normal ranges for 30 d old pigs as reported by Ventrella et al. (2017), indicating that there were no physiological problems feeding DTB to nursery pigs. Shields et al. (2011) had also used a blood biochemistry analysis, similar to this study, to evaluate the safety and toxicity of different inclusions of glycerol in nursery pig diets.

The jejunum and ileum functions in young pigs is often impaired in the post-weaning period because of the variety of stressors at this time (Moeser et al., 2017). The overall histomorphometry of the small intestinal in this study was not affected by the dietary treatments, expect for the lower inclusion of DTB resulted in deeper crypts in the ileum in Exp. 1. These results are consistent with previous work by Xu et al. (2017) who evaluated the effects of organic acids and essential oils for pigs at the end of nursery phase and no changes in the morphometry of small intestine were observed. On the other hand, these results contrast improvements in intestinal morphometry observed in tilapias fed DTB (*Oreochromis niloticus* × *O. aureus;* Liu et al., 2009). In this study, pigs were approximately 47 d old at the

time of tissue harvest and were given the same transition diet in first week post-weaning. Therefore, that implicates that weaning stressors were unlikely to be affecting intestinal parameters at this age. Despite the fact that animals were individually housed, any changes in intestinal health could be attributed to nutritional effects of the dietary treatments. In the present study, intraepithelial lymphocytes numerically linear decreased in the ileum while Goblet cells linear increased numerically in the same tissue as the DTB concentration increased in the diet. Taken together, this finding may indicates that DTB fed pigs had a quicker maturation of the ileum compared to CON pigs.

Goblet cells are responsible to establish a physical and biochemical barrier to luminal content with the intestinal epithelial and underlying immune cells by secreting highly glycosylated mucins and forming the mucus layer (Dupont et al., 2014). Regardless the absence of changes in morphometry, the increase in goblet cells observed in this study, indicates that pigs fed DTB had a better physical barrier in the ileum which is agreement with the lower number of intraepithelial lymphocytes infiltrated in the epithelium. Moreover, even though the overall WBC count was not affected by DTB, the proportion of monocytes was lower. This finding corroborates with a possible lower immunological activation and less monocytes/macrophages recruitment to sites of inflammation as previous described by Che et al. (2011) in pigs. Moreover, Jones et al. (2018) speculated that broilers fed DTB had lower levels of inflammation because of an observed decreasing plasma alpha-1 acid glycoprotein in the blood. Therefore, a possible immunomodulatory effect of DTB in the intestinal mucosa may explain one of the mechanisms of how growth performance was enhanced.

In this study, overall nutrient digestibility was not affected by inclusion of DTB in the diet expect for N. In other species, DTB have been reported to positively affect nutrient digestibility. Jones et al., (2018) showed that the inclusion of 0.5% of DTB in the diet of broilers improved Ca and P digestibilities. In addition, nutrients digestibilities improvements were followed by enhancements in bone mineralization and quality of DTB fed broilers (Pirzado et al., 2020b). The ileum digestibility of Ca was also improved by the inclusion of DTB in the diet of hens during molt and post-molt period, with lower Ca in the tibia but no effects on bone quality parameters, indicating better Ca absorption (Malheiros et al., 2018). Nevertheless, the lack of changes in nutrient digestibilities, especially trace minerals, implicates that DTB have no adverse effects upon competing for absorption with other minerals in the diet while improving growth performance. A better N utilization was

indicated in the Exp. 1 through the linear numerically decrease in BUN. In the Exp. 2, the N balance was positively affected by DTB, indicated by improvements in N digestibility which corroborates with greater growth performance and protein deposition in this group of pigs.

CONCLUSION

In summary, the inclusion of 0.5% of DTB in the diet of nursery pigs resulted in better growth performance in Exp.1 and the same inclusion had improved growth in Exp.2. Overall nutrient digestibilities were not affected by DTB in the diet, indicating that no mineral absorption competition had occur. The numerical decrease in blood urea N in Exp. 1 was followed by improvements in N digestibility in Exp. 2. It was observed that no indication of hepatic disfunction neither immunologic nor hematologic changes took place. The improvements in growth performance are likely to be related to DTB immunomodulatory effects in the intestine, better protein deposition rate, and possibly due to unknown biological functions of some trace minerals and rare earth elements. In conclusion, DTB in the diets of nursery pigs improved growth performance and N balance while not affected intestinal morphometry and metabolic parameters or mineral digestion. Specific functions of DTB components in young pigs need continued research into its true mode of action.

CONFLICT OF INTEREST

No conflicts of interest, financial, or otherwise are declared by the authors.

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Table 1. Average composition of the dacitic tuff breccia (DTB).

Item	%	ppm
Silicon dioxide, SiO_2	66.772	-
Alumina, Al_2O_3	12.35	-
Potassium oxide, K_2O	5.222	-
Calcium oxide, CaO	3.103	-
Sodium oxide, Na_2O	1.727	-
Iron oxide, Fe_2O_3	1.548	-
Magnesium oxide, MgO	0.971	-
Carbon, C	0.392	-
Titanium dioxide, TiO_2	0.215	-
Barium oxide, BaO	0.138	-
Manganese oxide, MnO_2	0.068	-
Phosphorus pentoxide, P_2O_5	0.043	-
Strontium oxide, SrO	0.024	-
Barium, Ba	-	414.1
Strontium, Sr	-	69.1
Lanthanum, La	-	61.0
Chlorine, Cl	-	57.6
Cerium, Ce	-	40.6
Florine, F	-	37.1
Neodymium, Nd	-	34.8
Rubidium, Rb	-	32.2
Zirconium, Zr	-	26.4
Lithium, Li	-	21.2
Zinc, Zn	-	14.1
Vanadium, V	-	13.162
Praseodymium, Pr	-	10.8
Lead, Pb	-	10.7
Boron, B	-	<10
Thorium, Th	-	8.6
Yttrium, Y	-	6.5
Samarium, Sm	-	5.1

Gallium, Ga	-	4.0
Gadolinium, Gd	-	3.5
Chromium, Cr	-	3.0
Dysprosium, Dy	-	2.9
Copper, Cu	-	2.3
Ytterbium, Yb	-	1.8
Erbium, Er	-	1.7
Molybdenum, Mo	-	1.6
Cesium, Cs	-	1.5
Scandium, Sc	-	1.5
Cobalt, Co	-	1.5
Nickel, Ni	-	1.3
Tantalum, Ta	-	1.3
Arsenic, As	-	1.2
Tin, Sn	-	1.1
Europium, Eu	-	1.0
Hafnium, Hf	-	0.7
Uranium, U	-	0.6
Holmium, Ho	-	0.6
Beryllium, Be	-	0.5
Terbium, Tb	-	0.5
Lutetium, Lu	-	0.3
Thulium, Tm	-	0.3
Selenium, Se	-	0.2
Tungsten, W	-	0.2
Bromine, Br	-	0.2
Bismuth, Bi	-	0.2
Thallium, Tl	-	0.2
Niobium, Nb	-	0.2

Trace minerals and elements <0.1 ppm: germanium (Ge), antimony (Sb), titanium (Ti), cadmium (Cd), indium (In), sulphur (S), silver (Ag), mercury (Hg), tellurium (Te), gold (Au), and rhenium (Re).

Table 2. Experiment 1 diet mycotoxin analysis (as-fed basis).

Phase	**Phase II**			**Phase III**		
Diet	**CON**	**DTB1**	**DTB2**	**CON**	**DTB1**	**DTB2**
Deoxynivalenol, ppm[1]	0.40	0.33	0.32	1.04	0.66	0.60
Aflatoxin, ppb	< 5	< 5	< 5	ND	ND	ND
Zearalenone, ppm	0.03	0.03	ND	0.03	0.04	0.03
Fumonisin, ppm	ND	ND	ND	ND	ND	ND

[1]Analysis of final diets was completed by Purdue University Animal Disease Diagnostic Laboratory by ELISA assays for each mycotoxin. ND = Not detected. The detection limit for Aflatoxin was 1.4 ppb and for Fumonisin was 0.2 ppm. Quantification limit for Aflatoxin was 5 ppb and for Fumonisin was 1 ppm.

Table 3. Experiment 2 diet mycotoxin analysis (as-fed basis).

Phase	**Phase II**		**Phase III**	
Diet	**CON**	**DTB1**	**CON**	**DTB1**
Deoxynivalenol, ppm[1]	0.59	0.45	0.70	0.60
Aflatoxin, ppb	ND	ND	< 5	ND
Zearalenone, ppm	ND	ND	0.03	0.04
Fumonisin, ppm	ND	ND	< 1	ND

[1]Analysis of final diets was completed by Purdue University Animal Disease Diagnostic Laboratory by ELISA assays for each mycotoxin. ND = Not detected. The detection limit for Aflatoxin was 1.4 ppb and for Fumonisin was 0.2 ppm. Quantification limit for Aflatoxin was 5 ppb and for Fumonisin was 1 ppm.

Table 4. Experiment 1 dietary composition of basal experimental diets (as-fed basis).

Item	**Phase 1**			**Phase 2**		
Ingredients, %	**CON**	**DTB1**	**DTB2**	**CON**	**DTB1**	**DTB2**
Corn	36.77	36.77	36.77	45.31	45.31	45.31
Soybean meal, 47.5% CP	16.48	16.48	16.48	21.46	21.46	21.46
Dried distillers grain with solubles	5.00	5.00	5.00	10.00	10.00	10.00
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00
Limestone	0.91	0.91	0.91	0.92	0.92	0.92
Monocalcium phosphate	0.12	0.12	0.12	0.38	0.38	0.38
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25
Trace mineral premix[2]	0.13	0.13	0.13	0.13	0.13	0.13
Se premix[3]	0.05	0.05	0.05	0.05	0.05	0.05
Salt	0.25	0.25	0.25	0.30	0.30	0.30
Spray dried plasma	2.50	2.50	2.50	0.00	0.00	0.00
Soy protein concentrate	4.00	4.00	4.00	2.50	2.50	2.50
Select menhaden fish meal	4.00	4.00	4.00	4.00	4.00	4.00
Dried whey	25.00	25.00	25.00	10.00	10.00	10.00
L-Lysine-HCl	0.20	0.20	0.20	0.27	0.27	0.27
DL-Methionine	0.08	0.08	0.08	0.07	0.07	0.07
L-Threonine	0.02	0.02	0.02	0.11	0.11	0.11
Mecadox10[4]	0.25	0.25	0.25	0.25	0.25	0.25
Corn premix	1.00	0.75	0.50	1.00	0.750	0.50
Dacitic tuff breccia premix[5]	0.00	0.25	0.50	0.00	0.250	0.50
Calculated values						
ME, kcal/kg	3427	3427	3427	3425	3425	3425
Crude protein, %	22.60	22.60	22.60	22.40	22.40	22.40
Calcium, %	0.85	0.85	0.85	0.80	0.80	0.80
Total P, %	0.66	0.66	0.66	0.65	0.65	0.65
ATTD P, %	0.46	0.46	0.46	0.41	0.41	0.41
SID[5] Lysine, %	1.37	1.37	1.37	1.27	1.27	1.27
SID Methionine, %	0.40	0.40	0.40	0.40	0.40	0.40
SID Met + Cys, %	0.72	0.72	0.72	0.69	0.69	0.69
SID Threonine, %	0.82	0.82	0.82	0.83	0.83	0.83

SID Tryptophan, %	0.24	0.24	0.24	0.22	0.22	0.22

[1]Provided per kg of diet: vitamin A, 6,614 IU; vitamin D3, 661 IU; vitamin E, 44.1 IU; vitamin K, 2.2 mg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg and B_{12} 38.6 µg.

[2]Provided per kg of diet: Fe, 121.3 mg; Zn, 121.2 mg; Mn, 15.0 mg; Cu, 11.3 mg; and I, 0.46 mg.

[3]Provided 0.3 ppm Se.

[4]Mecadox10 provided 55 ppm carbadox (Phibro Animal Health, Teaneck NJ, U.S.A).

[5]Dietary treatment premixes were mixed on Purdue campus prior to mixing into final diet. The premix contained the DTB minerals (0.25 or 0.50%) and mixed with fine ground corn.

[6]Standardized ileal-digestible.

Table 5. Experiment 2 dietary composition of basal experimental diets (as-fed basis).

Item	**Phase 1**		**Phase 2**	
Ingredients, %	**CON**	**DTB1**	**CON**	**DTB1**
Corn, 7.88% CP	35.17	35.17	46.32	46.32
Soybean meal, 47.5% CP	15.40	15.40	20.20	20.20
Dried distillers grain with solubles	5.00	5.00	10.00	10.00
Soybean oil	5.00	5.00	3.00	3.00
Limestone	0.97	0.97	1.11	1.11
Monocalcium phosphate	0.01	0.01	0.01	0.01
Vitamin premix[1]	0.25	0.25	0.25	0.25
Trace mineral premix[2]	0.13	0.13	0.13	0.13
Se premix[3]	0.05	0.05	0.05	0.05
Phtyase[4]	0.05	0.05	0.10	0.10
Salt	0.25	0.25	0.30	0.30
Spray dried plasma	2.50	2.50	-	-
Spray dried blood meal	1.50	1.50	-	-
Soy protein concentrate	3.00	3.00	2.50	2.50
Select menhaden fish meal	4.00	4.00	4.00	4.00
Dried whey	25.00	25.00	10.00	10.00
L-Lysine-HCl	0.20	0.20	0.35	0.35
DL-Methionine	0.20	0.20	0.15	0.15
L-Threonine	0.08	0.08	0.12	0.12
L-Tryptophan	0.02	0.02	0.03	0.03
ChlorMax 50[5]	0.00	0.00	0.40	0.40
Neo-Terramycin 100/100[6]	0.25	0.25	-	-
Chromium oxide	0.00	0.00	0.20	0.20
Corn premix[7]	1.00	0.50	0.80	0.30
Dacitic tuff breccia premix	0.00	0.50	0.00	0.50
Calculated values				
ME, kcal/kg	3506.2	3506.2	3403.2	3403.2
Crude protein, %	22.7	22.7	21.9	21.9
Calcium, %	0.85	0.85	0.80	0.80
Total P, %	0.61	0.61	0.56	0.56

ATTD P, %	0.40	0.40	0.34	0.34
Total Lysine, %	1.57	1.57	1.47	1.47
SID[8] Lysine, %	1.40	1.40	1.30	1.30
SID Methionine, %	0.50	0.50	0.47	0.47
SID Methionine + Cysteine, %	0.82	0.82	0.76	0.76
SID Threonine, %	0.87	0.87	0.81	0.81
SID Tryptophan, %	0.25	0.25	0.24	0.24
Analyzed values				
Dry matter, %	92.79	92.60	89.65	89.15
Ash, %	5.53	5.93	5.50	5.90
Gross energy, kcal/kg	4136	4102	4088	4021
Crude protein, %	21.18	19.86	22.00	22.13
NDF, %	-	-	7.65	7.96
ADF, %	-	-	4.14	4.31
Calcium, %	0.74	0.67	0.97	0.94
Total P, %	0.56	0.59	0.53	0.53
Cr,%	0.00	0.00	0.06	0.07
Zn, %	0.02	0.02	0.02	0.02
Na, %	0.272	0.230	0.182	0.173
Mg, %	0.21	0.24	0.31	0.29
K, %	0.07	0.05	0.04	0.07
Mn, %	0.01	0.01	0.01	0.01
Amino Acids*				
Lysine, %	-	-	1.55	1.53
Methionine, %	-	-	0.44	0.42
Cysteine, %			0.33	0.30
Threonine, %	-	-	0.91	0.87
Tryptophan, %	-	-	0.24	0.24
Isoleucine, %	-	-	0.95	0.90
Leucine, %	-	-	1.94	1.81
Valine, %	-	-	1.07	1.02

[1]Provided per kg of diet: vitamin A, 6,614 IU; vitamin D3, 661 IU; vitamin E, 44.1 IU; vitamin K, 2.2 mg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg and B_{12} 38.6 µg.

[2]Provided per kg of diet: Fe, 121.3 mg; Zn, 121.2 mg; Mn, 15.0 mg; Cu, 11.3 mg; and I, 0.46 mg.

[3]Provided 0.3 ppm Se.

[4]Provided 300 FTU per kg of the diet in phase 1 and 600 FTU/kg of the diet in phase 2 (Phyzyme, Danisco Animal Nutrition, Marlborough, UK).

[5]ChlorMax-50 provided 441 ppm chlortetracycline (Zoetis, Kalamazoo, MI, U.S.A).

[6]Neo-Terramycin provided 551 ppm of neomycinsulfate and 551 ppm oxytetracycline (Phibro Animal Health, Teaneck, NJ, U.SA).

[6]Standardized ileal-digestible

[7]Dietary treatment premixes were mixed on Purdue campus pior to mixing into final diet. The premix contained the DTB minerals (0.50%) and chromium oxide mixed with fine ground corn.

[8]Standardized ileal-digestible.

*Analysis conducted by University of Missouri Experiment Station Chemical Laboratories.

Table 6- Effect of DTB in the feed on nursery pig growth performance (Exp. 1)[1].

Item	**CON**	**DTB1**	**DTB2**	**SEM**	***P*-Value**
Initial age, days	27.3	28.0	27.8	0.48	0.5319
Initial BW, kg	6.82	6.74	6.81	0.223	0.3287
Final BW, kg	15.05	15.08	15.84	0.714	0.1092
ADG, grams	392	397	430	28.7	0.0679
ADFI, grams	591[b]	592[b]	669[a]	38.6	0.0083
G:F	0.655	0.663	0.644	0.023	0.7512

[a,b]Within a row, means with different letters differ by Tukey test (P < 0.05).

SEM: Standard error for the mean.

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON), 0.25% DTB inclusion (DTB1), and 0.50% DTB inclusion (DTB2).

Table 7 - Effect of DTB in the feed on nursery pig growth performance (Exp. 2)[1].

Performance	**CON**	**DTB**	**SEM**	***P*-Value**
Age, days	26.1	26.4	0.15	0.1411
Initial weight, kg	7.73	7.73	0.205	0.8957
0 to 7 days				
BW d 7, kg	9.74	9.65	0.299	0.6794
ADG, grams	287	275	25.2	0.6882
ADFI, grams	441	400	31.5	0.3567
G:F	0.643	0.707	0.0442	0.2688
7 to 14 days				
BW d 14, kg	10.63	11.77	0.438	0.0335
ADG, grams	128	302	46.5	0.0151
ADFI, grams	428	537	52.9	0.1378
G:F	0.275	0.608	0.0848	0.0178
14 to 20 days				
BW d 20, kg	13.30	14.77	0.596	0.0388
ADG, grams	444	500	36.2	0.1641
ADFI, grams	642	768	46.6	0.0144
G:F	0.675	0.644	0.0211	0.3274
0 to 20 days				
ADG, grams	279	352	27.1	0.0421
ADFI, grams	497	558	34.8	0.1603
G:F	0.562	0.624	0.0340	0.2245
Metabolism period				
Initial weigh, kg	11.10	12.47	0.492	0.0218
Final weigh, kg	12.28	13.75	0.540	0.0345
ADG, grams	396	427	42.0	0.6017
ADFI, grams	544	668	37.9	0.0262
G:F	0.725	0.642	0.0660	0.3160

SEM: Standard error for the mean.

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON) and 0.50% DTB inclusion (DTB).

Table 8 - Effect of DTB in the feed on nursery pig blood parameters (Exp. 1)[1].

Item	**CON**	**DTB1**	**DTB2**	**SEM**	***P*-Value**
Glucose, mg/dL	122.53	119.40	120.75	3.126	0.7777
Urea, mg/dL	9.27	8.80	7.75	0.544	0.1318
Creatinine, mg/dL	0.927	0.867	0.888	0.0305	0.3752
Biochemestry analysis					
Serum total protein, g/dL	5.00	4.88	5.08	0.105	0.3891
Plasma total protein, g/dL	5.58	5.45	5.48	0.078	0.5028
Albumin (A), g/dL	3.05	2.88	2.99	0.075	0.2937
Globulin (G), g/dL	1.95	2.00	2.09	0.080	0.4384
Aspartate aminotransferase, U/L	42.13	62.33	47.81	9.34	0.2965
Alkaline phosphatase, U/L	218.53	293.27	223.44	32.606	0.2017
γ-glutamyl transferase, U/L	45.53	54.53	43.25	4.356	0.1595
Minerals					
Phosphorus, mg/dL	9.32	8.75	9.16	0.277	0.3389
Calcium, mg/dL	10.88	10.85	11.03	0.117	0.5104
Sodium, mEq/L	138.00	139.07	139.25	0.630	0.3205
Potassium, mEq/L	6.35	6.29	6.34	0.224	0.9760
Chloride, mEq/L	101.07	101.27	101.69	0.720	0.8182
Magnesium, mEq/L	2.09	2.13	2.06	0.050	0.6488
Hematoligical analisys					
Red blood cells, 10^6/µL	7.06	6.58	6.79	0.166	0.1243
Hematocrit, %	36.08	35.17	36.43	1.033	0.6747
Hemoglobin, g/dL	10.88	10.57	11.07	0.314	0.5199
White blood cells (WBC), 10^3/µL	20.35	19.57	19.41	1.437	0.8802
Neutrophil:WBC, 10^3/µL	4.58	5.52	4.73	0.669	0.5627
Lymphocytes:WBC, 10^3/µL	14.27	12.98	13.03	1.175	0.6701
Monocytes:WBC, 10^3/µL	1.27[a]	0.79[b]	1.45[a]	0.171	0.0236

[a,b]Within a row, means with different letters differ by Tukey test ($P < 0.05$). SEM: Standard error for the mean.

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON), 0.25% DTB inclusion (DTB1), and 0.50% DTB inclusion (DTB2).

Table 9 - Effect of DTB in the feed on nursery pig's intestinal morphometry and number of epithelial cells (Exp. 1)[1].

Item	**CON**	**DTB1**	**DTB2**	**SEM**	***P*-Value**
Ileum					
Villous (V), µM	293.8	284.2	277.6	13.92	0.7134
Crypt (C), µM	250.7	261.3	253.2	14.17	0.8483
V:C ratio	1.14	1.11	1.10	0.040	0.8065
Goblet cells[2]	7.28	8.07	9.20	0.689	0.1678
Intraepithelial lymphocytes[2]	33.98	34.27	29.07	2.321	0.2373
Jejunum					
Villous (V), µM	297.8	310.5	304.7	18.22	0.8772
Crypt (C), µM	254.7[b]	292.0[a]	275.6[ab]	9.91	0.0401
V:C ratio	1.15	1.14	1.13	0.075	0.9771
Goblet cells	2.88	3.20	2.93	0.389	0.8261
Intraepithelial lymphocytes	42.73	43.11	39.72	2.669	0.6093

[a,b]Within a row, means with different letters differ by Tukey test ($P < 0.05$). SEM: Standard error for the mean of non-transformed data. P-Values for transformed data (log10).

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON), 0.25% DTB inclusion (DTB1), and 0.50% DTB inclusion (DTB2).

[2]Numbers are given as cells (goblet or intraepithelial lymphocytes)/100 epithelial cells.

Table 10 – Daily energy and N balance in nursery pigs fed diets containing DTB (dry mater basis; Exp. 2)[1].

Item	CON	DTB	SEM	P-value
ADG, grams	396	427	42.0	0.6017
ADFI, grams	544	668	37.9	0.0262
Dry matter, %	86.26	85.62	0.971	0.6398
GE consumed, kcal/d	2130.6	2624.1	164.30	0.0457
GE in feces, kcal/d	340.97	437.43	40.179	0.1430
GE in urine, kcal/d	2.59	2.68	0.334	0.8504
GE digestibility, %	84.23	84.16	1.058	0.9647
Calculated diet DE, kcal/kg	3443.3	3384.1	-	-
Energy metabolizability, %	84.11	84.06	1.058	0.9739
Calculated diet ME, kcal/kg	3438.4	3380.0	-	-
N intake, g/d	18.35	23.10	1.432	0.0287
N in feces, g/d	7.65	8.23	0.971	0.6749
N absorbed, g/d	10.70	14.87	1.501	0.0627
N digestibility, %	58.74	65.82	4.341	0.1268

SEM: Standard error for the mean.

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON) and 0.50% DTB inclusion (DTB).

Table 11 – Apparent total tract digestibility (%) by nursery pigs of minerals in diet containing DTB (exp. 2)[1].

Item	CON	DTB	SEM	P-value
Phosphorus, %	70.11	66.74	2.648	0.2900
Calcium, %	63.99	54.13	5.564	0.1177
Sodium, %	87.31	83.30	2.133	0.1891
Zinc, %	48.14	43.14	4.932	0.3946
Iron, %	51.67	45.78	3.640	0.2317
Manganese, %	43.82	39.17	8.477	0.4124

SEM: Standard error for the mean.

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON) and 0.50% DTB inclusion (DTB).

Table 12 – Frequencies of therapeutic medication treatments for diarrhea on nursery pigs fed diets containing DTB (exp. 2)[1].

Experiment 1	**CON**	**DTB1**	**DTB2**	**Chi-Square**
Received one treatment	18.8	43.8	31.3	0.3123
Received two treatments	12.5	12.5	6.3	0.7999
Received three treatments	0.0	6.3	0.0	0.3601
Experiment 2	**CON**		**DTB**	**Chi-Square**
Received one treatment	83.3		83.3	1.0000
Received two treatments	58.30		33.3	0.2191
Received three treatments	8.3		8.3	1.0000

[1]DTB: dacitic tuff breccia. Dietary treatments were basal diet (CON) and 0.50% DTB inclusion (DTB).

Figure 1 – Jejunal cross sections stained in Alcian Blue **(A),** immunohistochemical staining for CD3 protein detection for intraepithelial lymphocytes count **(B)**, and hematoxylin and eosin for intestinal morphometry **(C)**. The proportion of Goblet cells and intraepithelial lymphocytes were calculated as number of cells per 100 columnar cells.

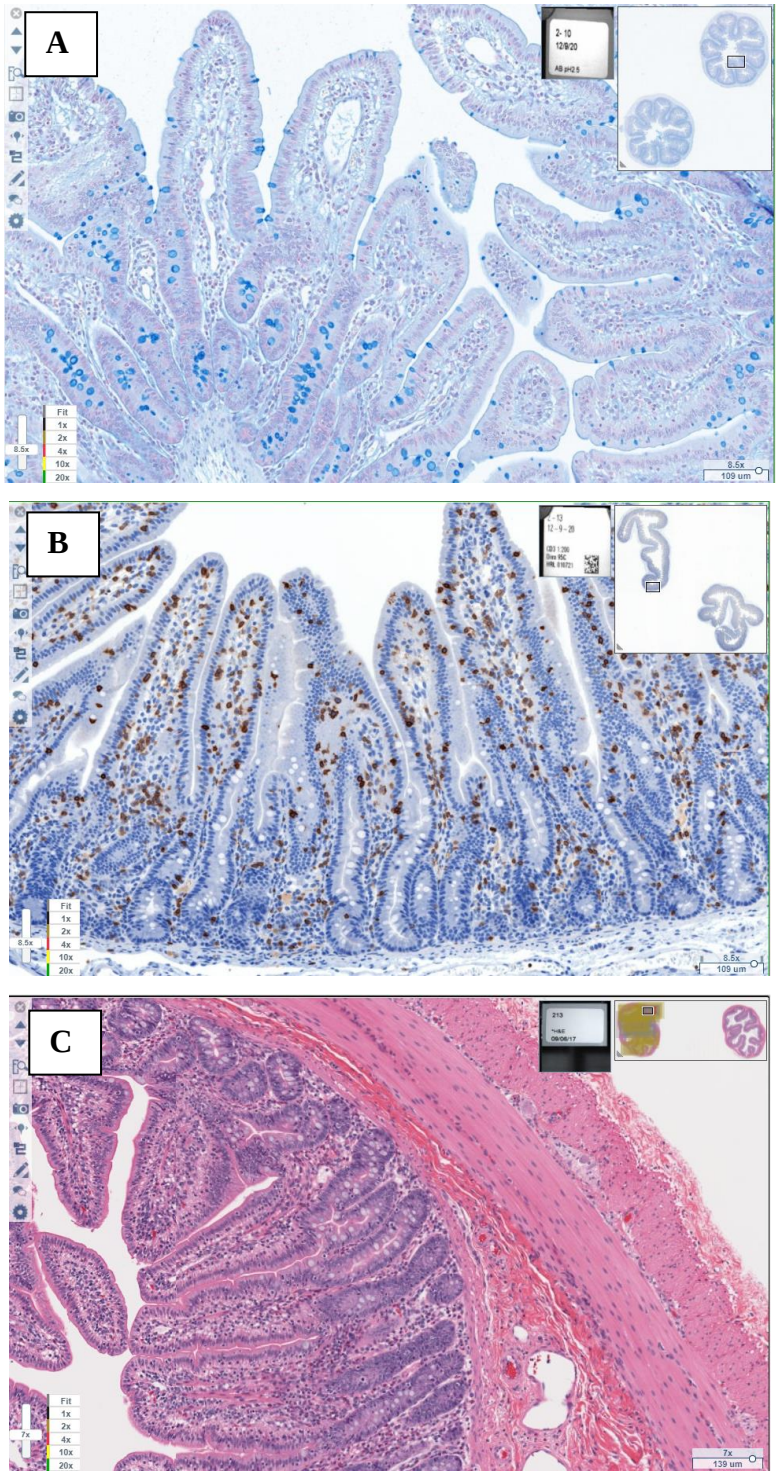

Figure 2 – Ileum cross sections stained in Alcian Blue **(A),** immunohistochemical staining for CD3 protein detection for intraepithelial lymphocytes count **(B)**, and hematoxylin and eosin for intestinal morphometry **(C)**. The proportion of Goblet cells and intraepithelial lymphocytes were calculated as number of cells per 100 columnar cells.