RHUAN FILIPE CHAVES

STRATEGIES TO ENSURE INTESTINAL AND SYSTEMIC HEALTH OF PIGLETS CHALLENGED WITH DIETS WITHOUT ANTIBIOTICS OR NUTRITIONAL DEFICIENCY AND HIGH DENSITY

LAVRAS - MG
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Orientador
Prof. Dr. Vinícius de Souza Cantarelli

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ESTRATÉGIAS PARA GARANTIR A SAÚDE INTESTINAL E SISTÊMICA DOS LEITÕES DESAFIOS COM DIETAS SEM ANTIBIÓTICOS OU DEFICIÊNCIA NUTRICIONAL E ALTA DENSIDADE

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 18 de março de 2022
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LAVRAS - MG
2022

A minha avó e família, meus pilares!

Maíra, minha melhor cúmplice!

Liz, minha inspiração!

Dedico

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À Deus, Aquele que caminha comigo, me guiando sempre para o melhor caminho.

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O presente trabalho foi realizado com apoio da Fundação de Amparo à Pesquisa de Minas gerais (FAPEMIG).

“Insanidade é continuar fazendo sempre a mesma coisa
e esperar resultados diferentes.”

Albert Einstein

RESUMO

O objetivo do estudo 1 foi avaliar os efeitos dos ácidos orgânicos associados aos polifenóis na resposta imune celular e humoral, histomorfologia, capacidade antioxidante na mucosa jejunal, produção de ácidos graxos de cadeira curta (AGCC) no ceco e microbiota colônica em leitões desmamados. Os tratamentos foram: dieta basal sem aditivos antimicrobianos (NC); dieta basal com 120 ppm de halquinol (PC); NC + 0,05% de blend com ácidos orgânicos associados a polifenóis (OAP1); NC + 0,10% da mesma mistura (OAP2). Na fase inicial II (dia 28 a 42), os suínos dos tratamentos PC e OAP2 apresentaram maior peso corporal (PC) e ganho de peso diário (GPD), assim como para o GPD de todo o período. Os suínos do NC tiveram a maior incidência de diarreia durante a fase inicial I e em relação ao período total OAP1 teve a menor incidência de diarreia. O grupo OAP2 reduziu a contagem de neutrófilos. Quando comparado com o grupo NC e PC, a suplementação de OAP2 aumentou significativamente a glutationa S-transferase (GST) na mucosa jejunal. Nas análises do microbioma, observou-se que os grupos OAP1 e NC apresentaram microbiota colônica semelhante quando comparados aos leitões PC e OAP2. Um aumento significativo no número de unidades taxonômicas operacionais (OTUs) foi observado no grupo NC. Em conclusão, a suplementação com 0,1% do blend com ácidos orgânicos associados a polifenóis pode substituir antibióticos promotores de crescimento e melhorar os parâmetros redox jejunais, modificar o microbioma do cólon e melhorar o desempenho de crescimento de leitões na fase de creche. O objetivo do estudo 2 foi avaliar a substituição do triptofano cristalino (CTrp) pela biomassa de triptofano (BTrp) e os efeitos no desempenho, escore fecal, permeabilidade intestinal, parâmetros redox, estado imunológico e cortisol de leitões criados em diferentes densidades de criação. Duas densidades de criação (0,15 e 0,40 m²/suíno) e três dietas (sem triptofano sintético (Trp), CTrp e BTrp) foram os fatores estudados. O peso corporal foi registrado aos 0, 8, 14, 28 e 42 dias. A pontuação fecal foi classificada como pontuação 1, pontuação 2 e pontuação 3 em todos os períodos experimentais. No dia 43, amostras de sangue foram coletadas para análises laboratoriais posteriores. Leitões alimentados com CTrp apresentaram maior peso corporal, CRD e GPD do que leitões dos tratamentos deficientes em Trp em todas as fases. Leitões que se alimentaram de BTrp tiveram maior peso corporal do que os tratamentos deficientes de Trp aos 28 e 42 dias e GPD a partir da fase pré-inicial II. Leitões que se alimentaram de BTrp apresentaram maior CRD apenas na fase inicial I. No período total, os leitões dos tratamentos deficientes em Trp apresentaram CRD e GPD mais baixos, e maior CA do que os leitões alimentados com fontes de Trp. A alta densidade de criação reduziu o peso corporal e GPD e aumentou a CA a partir da fase inicial I. Apenas o fator densidade de criação influenciou no escore fecal dos tratamentos. A partir da fase pré-inicial II, e considerando o período total, os leitões dos tratamentos com alta densidade de criação tiveram maior porcentagem de pontuação 1 do que os leitões mantidos em densidade normal de criação. A partir da fase iniciaal II, e considerando o período total, observou-se maior porcentagem de escore 2 nos tratamentos com densidade normal de criação. Houve interação dos fatores analisados (densidade de criação e Trp) para a concentração sérica de cortisol, de modo que os leitões do tratamento HD apresentaram maior concentração que os leitões do tratamento ND, assim como do tratamento HD+BTrp. Também houve interação para a concentração de IFN-alfa, sendo que o tratamento HD+BTrp apresentou concentração menor que os demais tratamentos, exceto para o tratamento ND+Ctrp. Em conclusão, a fonte convencional de triptofano pode ser substituída por triptofano de biomassa na fase de creche. Adicionalmente, o uso de biomassa de triptofano pode atenuar as concentrações de indicadores de estresse em animais em altas densidades de produção.

Palavras-chave: Aditivos. Leitões. Aminoácidos. Saúde intestinal.

ABSTRACT

The objective of study 1 was to evaluate the effects of organic acids associated with polyphenols on cellular and humoral immune response, morphology, antioxidant capacity in the jejunal mucosa, production of short-chair fatty acids (SCFA) in the cecum and colonic microbiota in weaned piglets. The treatments were: basal diet without antimicrobials additives (NC); basal diet with 120 ppm of halquinol (PC); NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); NC + 0.10% of same blend (OAP2). In the starter II phase (d 28 to 42), the pigs of the PC, and OAP2 treatments showed a greater body weight (BW) and average daily gain (ADG) as well as for the entire period, ADG. The NC pigs had the highest incidence of diarrhea during the starter I phase, regarding the overall period OAP1 had the lowest diarrhea incidence. The OAP2 group reduced the counts of neutrophils. When compared with the NC and PC group, OAP2 supplementation significantly increased the glutathione S-transferase (GST) in the jejunal mucosa. In the microbiome analyses, it was observed that the OAP1 and NC groups had similar colonic microbiota when compared to the PC and OAP2 piglets. A significant increase in the number of operational taxonomic units (OTUs) was observed in the NC group. In conclusion, supplementation with 0.1% of the blend with organic acids associated with polyphenols can replace growth promoting antibiotics and increase jejunal redox parameters, modify the colon microbiome and improve the growth performance of piglets in the nursery phase. The objective of study 2 was to evaluate the replacement of crystalline tryptophan (CTrp) by tryptophan biomass (BTrp) and the effects on growth performance, score fecal, intestinal permeability, redox parameters, immune status, and cortisol of piglets raised at different rearing densities. Two rearing density (0.15 and 0.4 m2/pig) and three diets (without synthetic tryptophan (Trp), CTrp and BTrp) were the factors studied. The body weight was recorded at 0, 8, 14, 28, and 42 days. Fecal scoring was graded as score 1, score 2, and score 3 all the experimental periods. On day 43, blood samples were collected for further laboratory analyses. Piglets that fed CTrp had higher BW, ADFI, and ADG than piglets from the Trp-deficient treatments at all phases. Piglets that fed BTrp had higher BW than the Trp-deficient treatments at 28 and 42 days and ADG from the pre-starter II phase. Piglets that fed BTrp had higher ADFI only in the starter I phase. In the total period, piglets from Trp-deficient treatments had lower ADFI and ADG, and higher FCR than piglets that fed Trp sources. The high rearing density reduced the BW and ADG and increased the FCR from the starter I phase. Only the rearing density factor influenced the fecal score of the treatments. From pre-starter II, and considering the total period, piglets from treatments with high rearing density had a higher percentage of score 1 than piglets that were kept at normal rearing density. From starter II, and considering the total period, a higher percentage of score 2 was observed in treatments with normal rearing density. There was an interaction for the analyzed factors (rearing density and Trp) for the serum cortisol concentration, so that the piglets of HD treatment had higher concentration than piglets of ND treatment, as well as HD+BTrp treatment. There was also interaction for the concentration of IFN-alpha, and the HD+BTrp treatment had a lower concentration than the other treatments, except for the ND+Ctrp treatment. In conclusion, the conventional tryptophan source can be replaced by biomass tryptophan in the nursery phase. Additionally, the use of tryptophan biomass can attenuate the concentrations of stress indicators in animals at high rearing densities.

Keywords: Additives. Piglets. Amin acids. Intestinal health.

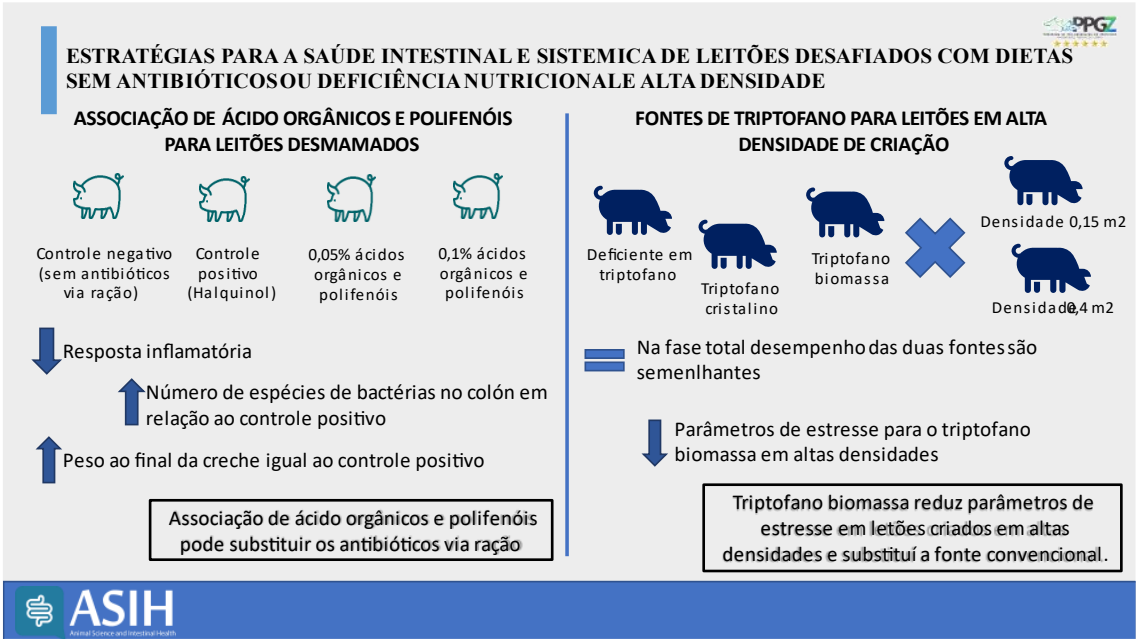
Estratégias para garantir a saúde intestinal e sistêmica dos leitões desafios com dietas sem antibióticos ou deficiência nutricional e alta densidade

Elaborado por **Rhuan Filipe Chaves** e orientado por **Vinícius de Souza Cantarelli**

Estratégias nutricionais para minimizar o uso de antibióticos promotores de crescimento e o estresse na produção suína têm sido alvo de diversos estudos. Neste caso, são usados aditivos nutricionais que podem substituir estes antibióticos assim como reduzir o estresse da superlotação nas baias.

No experimento 1, uma associação de ácido orgânicos e polifenóis foi avaliada como alternativa ao uso de antibióticos promotores de crescimento na ração. Foi observado que o uso do aditivo foi capaz de melhorar a atividade de enzima antioxidante e modificou o número de espécies de bactérias presentes no colón dos leitões recém-desmamados. Estes leitões apresentaram o mesmo peso na saída de creche que os animais tratados com o promotor de crescimento. Como os resultados de desempenho foram semelhantes entre os suínos que receberam antibióticos, podemos concluir que a associação de ácido orgânicos e polifenóis funciona com substituto ao antibiótico promotor de crescimento.

No experimento 2, foram avaliadas duas fontes de triptofano em duas diferentes densidades de criação na fase de creche. Uma das fontes testadas é comumente usada na dieta de suínos e a outra é uma nova forma. Na fase total, não houve queda no desenvolvimento dos suínos quando as fontes de triptofano foram incluídas. A maior densidade de animais prejudica o desempenho. A fonte alternativa melhorou os biomarcadores de estresse no sangue nos animais em alta densidade. Desta forma podemos concluir que a fonte alternativa não prejudica o desempenho e ajuda a minimizar o estresse de animais em alta densidade de criação.



Estratégias nutricionais para minimizar o uso de antibióticos promotores de crescimento e o estresse na produção suína têm sido alvo de diversos estudos. Dois experimentos foram conduzidos um para avaliar a substituição de antibióticos promotores de crescimento e outro para avaliar a redução do estresse com aminoácidos. O uso de ácidos orgânicos e polifenóis substitui antibióticos e o uso de triptofano de fonte alternativa substitui o convenciona e melhora parâmetros de estresse em leitões em altas densidades.

Tese de doutorado em Zootecnia na UFLA, defendida em 18/03/2022.

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CHAPTER 1

1 INTRODUCTION

The world population will grow in the coming years as will the demand for animal protein. Thus, improvements in the production system aiming productivity are of importance to meet this demand. For this to happen, new technologies need to be developed and more applied research that adds value to the production chain needs to be carried out.

The nursery period, within the swine production system, is characterized as one of the most challenging. In the growth curve of piglets, in commercial situations, in the post-weaning period there is a drop in growth, reaching loss of body weight. There are numerous factors that lead to this phenomenon, including separation from the mother, change of environment, formation of a new social group, change in diet, sanitary pressure, among others. All these factors are highly stressful for the piglet in the first days after weaning and depending on the intensity in which the stress occurs, the effects are irreversible due to the high plasticity that the animals still have in this period. The stress caused by these multiple factors causes an imbalance in the microbiota and immune system response, harming the intestinal and integral health of the animals.

In order to alleviate the deleterious effects of weaning, antibiotics were widely used for many years, but in recent years the pressure to withdraw or reduce antibiotics via feed has been growing, which creates an opening for the emergence of new technologies that can replace these compounds. However, care in this phase is not limited to the first days of nursery and the use of antibiotics, other factors as the animals grow can contribute to performance losses. It is not uncommon to find situations in which there is overcrowding of animals, low availability of feeders and high temperatures inside the facilities, factors that are still poorly studied and that lead to the development of chronic stress in animals and, therefore, growth performance losses.

In this context, develop new technologies that can replace the use of growth-promoting antibiotics, as well as help the return of homeostasis in animals in times of intense stress. This study can make a solid contribution to the use of additives as an alternative to growth promoters as well as new sources of amino acids with additive effects in the nursery phase.

2 LITERATURE REVIEW

2.1 Challenges in the nursery phase

The swine nursery phase is one of the most complex in the management of swine production. The first days at the nursery are the most critical and, therefore, dedication must be intense so that the litter adapts to the new system. In addition to the difficulty in the feed transition, the separation of the sow and the change of environment, a new social conformation is established by the mixing of batches and often with elevated densities in the pens. All these factors, added to the conditions of the new facility, make the nursery period the most challenging due to the high stress and health challenges to the herd.

2.1.1 Weaning

In nature, weaning is a gradual process that occurs around 12 to 17 weeks, a period that coincides with the almost complete development of the gastrointestinal tract (TGI) and the immune system (MOESER; POHL; RAJPUT, 2017). In commercial pig farms, the weaning age decreased over the years (YANG et al., 2016), in order to increase productivity and today this process occurs in about three weeks. Early weaning around 21 days, practiced in most farms, produced productivity gains by increasing pigs/sow/year production and facility optimization (BUTLER et al., 2008). However, early weaning in production systems occurs at the time when the barrier of the gastrointestinal tract still in development (POHL; MEDLAND; MOESER, 2015), resulting in late maturation and intestinal barrier dysfunction, reduction in weight gain, and increased susceptibility to enteric diseases (MOESER; POHL; RAJPUT, 2017). The impact of weaning stressors not only causes physiological changes in the intestine but is also related to immunological changes and microbiota composition in early weaned piglets compared to later weaning (PLUSKE, 2013). Physical and functional gastrointestinal changes in early weaned pigs have short- and long-term effects and may persist into adulthood (MOESER; POHL; RAJPUT, 2017). Weaning stressors are intrinsic to this period and will affect all piglets at this stage, however, weaned piglets between four and five weeks of age have better development and function of the TGI and the immune system, which can have a beneficial impact on piglets in the post-weaning period (MARSI et al., 2015; XUN et al., 2018).

Weaning has consequences for the whole gastrointestinal tract (PLUSKE, 2016). However, the small intestine has been reported as the most affected part during this process, responding with anatomical, physiological, and immunological adjustments to adapt the changes that occurred due to the stress in this period (WIJTTEN; LANGHOUT; VERSTEGEN, 2012; PLUSKE, 2013; BURKEY; SKJOLAAS; MINTON, 2009). The turnover of the intestinal epithelium in piglets occurs rapidly, every three or four days, and this process requires a high energy amount (DANIEL et al., 2014). As a consequence of low feed intake after weaning, the intestinal structure responds with changes in its morphology and barrier function (WIJTTEN; VAN DER MEULEN; VERSTEGEN, 2011). Another important fact is that intestinal invasion by pathogens at this stage also causes damage to the intestine, and epithelial cells respond by increasing their cell turnover rate, impacting the architecture of villi and crypts (HU et al., 2013). As a consequence of changes in villi and crypts, the villous:crypt relationship is significantly lower in weaned piglets than in non-weaned piglets (PLUSKE et al., 2003). According to Hampson (1986) and Li et al. (1991), the damage that occurs in the intestinal epithelium is transient but may persist for two weeks after weaning. Weaning age is an important factor related to the severity of the intestinal barrier injury after weaning (MOESER; POHL; RAJPUT, 2017), influencing the recovery period of villi atrophy (PLUSKE et al., 2003). Evaluating the influence of weaning at 17, 21, 28, and 35 days of age on changes in intestinal development, Gu, Li, and She (2002) observed that at five days after weaning villi atrophy was greater in piglets weaned at 17 days and took 11 days to recover the architecture compared to other ages. However, in animals weaned at 28 days there was no reduction in villi height and 15 days after weaning there was an increase of 111% in villi height.

Age at weaning also plays a critical role in intestinal permeability, which can compromise barrier function and favor the translocation of pathogens. Evaluating weaning at 19 and 28 days of age on intestinal function 24 hours after weaning, Moeser et al. (2007) observed that weaning at 19 days induced acute disturbances in intestinal permeability in the jejunum and colon, marked by reduced transepithelial electrical resistance (TER) and increased mannitol flow between tight junctions compared to piglets weaned at 28 days. Pohl et al. (2017) in a study that evaluated the effect of age at weaning at 15 days compared to 28 days, found that 15-day piglets had diarrhea most of the time (43.6%) compared to 28 (4.80%). For ileal permeability, younger weaning showed an increase in mannitol flow rate

and decrease in TER compared to older piglets, both at 49 days and 140 days of age, showing a lasting effect of weaning on intestinal barrier permeability.

Smith et al. (2010) investigated the immune response in the jejunum of piglets weaned at 15 and 28 days of age and demonstrated that the jejunal mucosa of early animals presented a greater density of cellular infiltrates (lymphocytes, macrophages, and neutrophils) on the lamina itself in relation to late weaning, suggesting increased inflammation and antigenic stimulation in the mucosa, due to impaired barrier function because of increased intestinal permeability. Besides, Moeser et al. (2007), Smith et al. (2010), McLamb et al. (2013), and Pohl et al. (2017) evaluated the activation of mast cells in the intestinal mucosa by the effect of weaning stress and concluded that, as the age of weaning decreases, the number of degranulated mast cells increases. Mast cells activate the release of mediators such as histamine and proteases that can induce injury to the intestinal barrier and activate enteric secretory neurons that trigger the pathophysiological mechanisms of diarrhea (OVERMAN et al., 2012). Mast cells also interact with corticotropin release factor (CRF) receptor 1, which is increased in the intestinal mucosa of prematurely weaned piglets and triggers immediate and long-term disturbances in the structure of the intestinal barrier (McLAMB et al., 2013; MEDLAND et al., 2016). Thus, consequent events of stress caused by weaning significantly interfere in the absorptive and secretory function of the gastrointestinal tract as the age of weaning decreases and these impacts may extend to subsequent stages, where the animals may present a lower-than-expected development (HU et al., 2013). Therefore, age at weaning, besides assisting in weaning weight and improving consumption, contributes to improved intestinal health of animals and therefore improves post weaning performance.

2.1.2 Rearing density

The increasing intensification in the swine production system due to genetic and nutrition improvements brought with it an increase in the number of animals produced. However, expanding facilities has a high cost, which in many cases leads to overcrowding of facilities. In the literature, studies with rearing density for piglets in the nursery phase and the performance response are still inconsistent and some have confounding factors that make it difficult to accurately conclude the effect.

Density can be defined as the number of animals raised per m^2 of floor. In the literature we found some variations to define the ideal stocking density. In the study by Gonyou et al. (2006) it was found that the gain is maximized when the floor space allowance

is equal $k \times BW^{0.667}$ where *k* represents a space allowance coefficient. Following the allowances stipulated by the European Council Directive (2008), the *k* values for pigs with 20 and 30 kg are, respectively, 0.041 and 0.031. Another variant of this equation was suggested by the Code of Practice for Pigs, where the recommended floor space for pigs is $0.035 m^2/kg$. Variables such as temperature, floor type, and group size change the value of *k* (NATIONAL FARM ANIMAL CARE COUNCIL, 2014). Pigs exposed to high temperatures tend to stay inactive longer, that is, they reduce the contact between them and look for cooler places inside the pens (SPOOLDER et al., 2012). When swine are kept in thermoneutral conditions, the value of k used as a basis to determine the area is 0.036, and for swine kept at room temperature above 25°C, the value of the constant should be 0.047, as the animals spend more time lying down and need more space (EFSA, 2005). Adopting a *k* constant lower than 0.033 for the nursery phase impairs the animals' performance (GONYOU et al., 2006).

The reduction in piglet housing space can affect the performance and consequently the economic viability of the system (WOLTER et al., 2000a; JENSEN; STUDNITZ; PEDERSEN, 2010). The increase in rearing density in the nursery can lead to loss of growth performance (YEN; POND, 1987; WOLTER et al., 2000b; BRUMM et al., 2001; WOLTER et al., 2003b) and worsen health problems once which leads to greater accumulation of manure in the environment (HACKER et al., 1994). According to Gonyou et al. (2006), a 3% reduction in housing space can result in a 1% drop in feed intake and weight gain in piglets. Thus, Patience et al. (2004) observed a reduction in the weight gain of piglets during the nursery phase housed at 0.25 m²/animal when compared to animals housed at 0.30 and 0.35 m²/animal. The negative effect of high densities on average daily gain (ADG) has already been reported by several authors (YEN; POND, 1987; WOLTER et al., 2000b; BRUMM et al., 2001; WOLTER et al., 2003b). However, studies investigating the effect of density on feed intake and feed conversion ratio (FCR) in nursery have shown inconsistent results. Brumm et al. (2001) and Wolter et al. (2003b) found a reduction in consumption when comparing two densities, however Yen and Pond (1987) found results only when the stocking was changed along with the density. The reduction in the feed intake at high densities can result in a worse FCR (WOLTER et al., 2000b; WOLTER et al., 2003b). The reduction in ADG has also been identified as responsible for the poor FCR in animals with space restriction (YEN; POND, 1987). However, Brumm et al. (2001) found no difference in feed conversion of piglets housed at higher densities, even though they showed a worsening in weight gain and feed consumption. Another relevant aspect that can help to mitigate the

negative effect of density is the availability of feeders. The combined effects of density and feeder space have already been reported (WOLTER et al., 2002), however, this interaction has not yet been well elucidated. Most of the works that use different densities end up changing the feeder space to serve the animals. (WOLTER et al., 2002; WOLTER et al., 2003a). Feeding space restriction may be one of the main reasons for reduced feed consumption when animals are subjected to high densities (GONYOU, 1999).

It is important to mention that high stocking density has stressful effects on animals and can alter gut health parameters. Although little research has been published in this area, current methods for quantifying stress in swine are known to rely on observable markers and therefore are not suitable for use with a large sample size (MARCO-RAMELL et al., 2016; SCOLLO et al., 2014). Piglets exposed to high densities had higher plasma protein oxidation and higher levels of stress markers than those kept at normal density (MARCO-RAMELL et al., 2011). On the gut microbiota, the impact of animal density has not been as widely studied as the impact on growth and welfare (FU et al., 2016; MARCO-RAMELL et al., 2011).

Most of the rearing density studies currently available in the literature are studies carried out in the growing and finishing phase. There is still a lack of information in the literature that explores the effects of rearing density in the nursery phase, mainly related to stress and intestinal health.

2.2 Immune system and gut health

The gastrointestinal tract functions as the main link between the environment and the organism. Site of numerous antigenic interactions, the result of constant contact with commensal and pathogenic microorganisms, and not least the contact with molecules derived from ingested food. Thus, it is essential to consider the responses at this location, avoiding an exacerbated and harmful reaction to the organism and simultaneously promoting an effective response against pathogenic antigens, when necessary. The good relationship between the intestinal microbiota and the immune system is essential for the health of its host. Furthermore, it has recently become obvious that alterations in intestinal microbial communities can cause immune dysregulation, leading to disorders that impair intestinal and systemic health.

2.2.1 Immune system associated with intestinal mucosa

The intestinal epithelium is composed of enterocytes and other specialized cells, connected by tight junctions, forming a barrier with selective permeability from the lumen to the internal environment (ORIÁ; BRITO, 2016), also contains several immune cells, including DCs, T cells, B cells, macrophages, and mast cells, these act in conjunction with intestinal epithelial cells to maintain intestinal homeostasis (ERI; CHIEPPA, 2013). Another important component is mucus, which covers the entire intestinal mucosa and plays a fundamental role in protecting the intestine against microorganisms and self-digestion (BIRCHENOUGH et al., 2015). Mucus serves as a first line of innate defense and is produced by superficial goblet cells, other epithelial cells and glands that are closely linked to the innate and adaptive immune system (PETERSON; ARTIS, 2014).

The differences in the functionality of each group of cells is what ensures the maximization of intestinal protection. Enteroendocrine cells are responsible for the production of numerous hormonal regulators that act on food digestion (GRIBBLE; REIMANN, 2016). The goblet cells mainly produce mucin, just as Paneth cells secrete antimicrobial peptides, thus establishing a physical and biochemical protection, respectively, against potentially harmful agents to the body and assisting in maintaining the homeostasis of the inflammatory response (ZHANG; HORNEF; DUPONT, 2015).

Specialized epithelial cells (IECs) can express pattern-recognition receptors (PRRs) that allow them to act as dynamic sensors of the microbial environment and as active participants in the targeting of immune mucosal cell responses (KAGNOFF, 2014). Members of the toll-like receptor families (TLRs), NOD (NLRs) and RIG (RLRs) provide distinct pathways for the recognition of microbial ligands or endogenous signs associated with pathogenesis (ABREU, 2010; ELINAV; HENAO-MEJIA; FLAVELL, 2013; BROQUET et al., 2011). Unlike other sites in the body (which are sterile), where the recognition of something not proper generates a highly inflammatory response, in the intestine due to resident microbiota the response should be changed. Although studies on PRR pathways in hematopoietic cells have focused mainly on their pro-inflammatory and antigen-presenting properties, its role in the regulation of tissue homeostasis and immunological tolerance has emerged as one of the main components of its function in IECs (PETERSON; ARTIS, 2014).

Another point that arouses interest is the reactive oxygen species (ROS), produced by the immune system in response to comatose or pathogenic bacteria that act on the intrinsic signaling of IECs that reflects in the repair of the epithelium, regardless of its bactericidal effects (LEONI et al., 2012). The mechanism of action is through the inactivation of redox-

sensitive tyrosine phosphatases, ROS promotes the formation of focal adhesions by IECs, necessary for cell migration and wound healing (LEONI et al., 2012).

Innate immune pathways, in addition to maintaining the hyperresponsiveness of IECs, must differentiate the signs derived from compressed and pathogenic microorganisms to scale an appropriate inflammatory response (MONTICELLI et al., 2011). The polarized nature of the intestinal epithelium allows the anatomical segregation of PRRs, proof of this is that in vitro and in vivo models demonstrated differential responsiveness of IECs to apical versus basolateral stimulation with multiple TLR ligands (LEE et al., 2006). However, the mechanisms involved in tolerance to microbial signals by IECs in situations of pathogen infection are still poorly defined. In contrast to sterile sites in the body, control of inflammation in the intestine may be better adapted to rely on the recognition of signs of danger from pathogenic bacteria (MATZINGER, 2002). Hazard recognition has been proposed to be measured by detecting properties associated with microbial viability, called viability-associated PAMPs (vita-PAMPs), which distinguish living pathogens from inert microbial debris, as well as by detecting virulence factors of pathogens, such as bacterial secretion systems and toxins that penetrate the cellular cytosol (SANDER et al., 2011). Although these mechanisms have been studied and identified in phagocytes and antigen-presenting cells, their function and relevance in IEC are less well understood.

Recently, a population of innate immune cells called innate lymphoid cells (ILCs) has been identified, and it has been found that these play a crucial role in intestinal immune homeostasis (PETERSON; ARTIS, 2014). ILCs express subunits of cytokine receptors, but unlike adaptive lymphocytes, they do not have expression of antigenic receptors summed up and, therefore, do not present any degree of antigenic specificity (ARTIS; SPITS, 2015). They are found on barrier surfaces, where they function as regulators of tissue homeostasis, inflammation, and early innate response to infection. ILCs are regulated, in part, by immunoregulatory signals derived from epithelial cells and exhibit phenotypic and functional heterogeneity (SPITS et al., 2013). They are characterized by their requirements for development and differential expression of cytokines in group 1, group 2, and group 3 ILCs, which share functional similarities with CD4+ Th1, Th2 e Th17 adaptive cell populations, respectively (SPITS; CUPEDO, 2012). The ILCs of the three groups are located mainly in mucosal-associated tissues, while natural killer (NK) cells are preferably located in secondary lymphoid organs (ARTIS; SPITS, 2015). Group 1 ILCs include classical NK cells and innate lymphoid cell 1 cells (ILC1) and are characterized by the production of Cytokines associated

with Th1 cells such as interferon γ (IFNγ) and tumor necrosis factor (TNF) in response to IL-12 and IL-15 (SPITS et al., 2013). Although NK cells can directly destroy target cells through cytotoxic activity, other ILC1 are limited to cytokine production in response to stimulation (FUCHS et al., 2013). ILC1 is found in approximately equal numbers in the small and large intestines (MOWAT; AGACE, 2014). Group 2 ILCs (collectively ilc2) produce IL-5 and IL-13 cytokines associated with Th2 cells (SPITS et al., 2013). These factors contribute to an early innate response to intestinal helminth infection and invoke a protective epithelial response, including goblet cell hyperplasia and increased mucus secretion. The proliferation and activation of ILC2 are supported by IL-25, IL-33 and TSLP molecules, derived from predominantly epithelial cells (NEILL et al., 2010), as well as cytokines such as IL-2, IL-4, IL-7 and IL-9 or lipid mediators such as prostaglandin D2 or leukotrien D4 (KLOSE; ARTIS, 2016). The contribution of microbial stimulation to these signals reinforces the idea of the epithelium as an integrator of environmental signals for the regulation of immune cell function (KABAT; SRINIVASAN; MALOY, 2014). Group 3 ILCs produce cytokines associated with Th17 and Th22 cells, including IL-17A and IL-22, in response to IL-23 stimulation (SPITS et al., 2013). This group includes ILC3, as well as lymphoid tissue-inducing cells, which have a well-established role in the organogenesis of secondary lymphoid tissue. IL-22 plays an important role in the protection of the intestinal epithelium after injury or infection by pathogens (ZENEWICZ et al., 2008). In addition, IL-22 derived from ILC3 supports anatomical containment of bacteria residing in lymphoid tissue associated with the intestine. The number of ILC3 also seems to increase from the proximal to the distal small intestine, which is consistent with the differences in bacterial density along this axis and with the fact that many of its functions seem to be directed to bacterial defense (MOWAT; AGACE, 2014).

Specialized cells known as intraepithelial lymphocytes (IELs) exist in close contact with the IECs layer, and two-way interactions between IELs and IECs keep immune homeostasis in the intestinal barrier (EDELBLUM et al., 2012). IELs exhibit an activated phenotype and include conventional T cells, as well as subsets of cells that express a restricted repertoire of T-cell receptor specificities and specialized properties, including Tγδ cells and NK cells (PETERSON; ARTIS, 2014). Compromised $CD4^+$ T cells may undergo transcriptional reprogramming when they become IELs to develop a distinct phenotype resembling $CD8^+$ cytotoxic T cells (MUCIDA et al., 2013). Although the influence of the local environment in promoting this development allocated change has not been explored, the

intimate interactions these cells have with The IECs suggest that signals derived from epithelial cells can promote their maintenance and function.

Additionally, some molecules become fundamental about the protection of the intestinal barrier against microorganisms, among them we can mention IgA, highly concentrated in the mucus of the duodenum and ileum, and antimicrobial proteins (AMPs). IgA is produced through the maturation of virgin B cells in secretory mature plasma cells. This is due to recombination of heavy chain class change (CSR) depends on the stimulus by mucosal DCs, which have the antigen and living bacteria of the intestinal epithelium (CERUTTI, 2008). Like the priming of a mucosal T-cell phenotype, these DCs are conditioned by signals derived from IECs to promote the exchange of Class IgA and a gut homing phenotype through the production of nitric oxide, IL-10 and retinoic acid, together with tgfβ signaling (MORA et al., 2006).

Another group of important cells in the maintenance of intestinal homeostasis are mononuclear phagocytes. These are represented by macrophages, DCs and mast cells, which are responsible for the uptake and presentation of antigens in the intestine (MOWAT; AGACE, 2014). These are scattered throughout the lamina itself, and are located in associated lymphoid tissues, including Peyer Plates and ILFs (GROSS; SALAME; JUNG, 2015).

Macrophages have the function of phagocyte and degrade microorganisms and dead tissue cells, as well as producing mediators that boost the renewal of epithelial cells and thereby have relevant importance in the promotion of intestinal homeostasis (GROSS; SALAME; JUNG, 2015). In addition, they produce large amounts of IL-10, which not only prevents inflammation by blocking pro-inflammatory responses (UEDA et al., 2010), but also promotes the survival and functions of local regulatory T cells (Treg) FoxP3+ in the mucosa (HADIS et al., 2011). The production of IL-1β by resident macrophages in response to the microbiota may have a similar ability to maintain th17 cell activity in the small intestine at steady state (SHAW et al., 2012). These properties are quite specific to the intestine and, unlike many other tissue macrophages, those of the small intestine and colon mucosa are derived by the continuous replacement of blood monocytes that differ locally under the control of the mucous environment (BAIN et al., 2014). In all parts of the intestine, macrophages are found relatively close to the epithelial surface, but this seems to be more prominent in the colon. Despite this, no marked functional difference was reported between the macrophages of the small intestine and colon (PETERSON; ARTIS, 2014). Thus, similar

processes seem to control the development and functions of macrophages throughout the intestine, despite presenting different markers and receptors (MOWAT; AGACE, 2014).

Dendritic cells specialize in t-cell communication, inhibiting self-activity through tolerogenic mechanisms, and activating T-cell immunity in response to threats (GROSS; SALAME; JUNG, 2015). CD103+, present in the lamina itself and associated with the intestinal epithelium that covers villi, promote surveillance of the luminal environment. They detect strange and inflammatory signals, acquire, and present antigens, and interact with T cells migrating to secondary lymphoid organs (BEKIARIS; PERSSON; AGACE, 2014). As t-cell activation is restricted to lymphoid tissues, intestinal DCs are forced to migrate from the lamina itself to the mesenteric lymph nodes (MLNs), or within the Peyer plates, to t-cell zones. Consequently, the expression of CCR7 is super regulated in the CDs before its migration from tissue to MLNs (JANG et al., 2006).

Although usually associated with allergic reactions and responses to parasites, eosinophils and mast cells are surprisingly abundant in the normal intestinal mucosa, indicating that they probably have important physiological roles (ROTHKOTTER; KIRCHHOFF; PABST, 1994). Eosinophils are believed to be important for tissue repair both at steady state and during inflammation in the small and large intestines (LAMPINEN et al., 2013). They may be important for the exchange of IgA class in Peyer's plates and to maintain the number of Plasma Cells IgA+, CD103+ DCs and Treg FoxP3+ cells in the small bowel lamina itself. These homeostatic effects seem to reflect the ability of eosinophils to produce TGF-β activating metalloproteinases (CHU et al., 2014).

Mast cells are found throughout the gastrointestinal tract, mainly in the lamina itself and in the submucosa, although there are also some in the epithelium (YU; PERDUE, 2001). They produce mediators that regulate epithelial barrier integrity, peristalsis, vascular tone and permeability, and there are important two-way interactions between mast cells and the local nervous system (BISCHOFF, 2009). Mast cells can be functionally distinct at different intestinal sites. In the small intestine produce a single protease dependent on TGF-β which is thought to be involved in tissue remodeling, while the colon contains more mast cells of the pro-inflammatory connective tissue (YU; PERDUE, 2001).

2.2.2 Modulation of tight junctions by the components of the immune system of the intestinal mucosa

Tight junctions (TJs) ensure epithelial and endothelial barrier functionality. This structure is protected by multiprotein complexes that seal the space between adjacent cells. These structures are composed of three groups of integral membrane proteins (claudins, occludins, and junctional binding molecules), as well as a considerable number of other accessory cytoplasmic proteins, which include zonula occluden (ZO), cingulin and other membrane-associated guanylate kinase proteins (ORIÁ; BRITO, 2016).

Therefore, any cytokine-mediated disturbance affecting the functionality of TJs results in increased paracellular permeability and increases tissue exposure to luminal antigens in organ systems such as the gastrointestinal and respiratory tracts. Although there is a gap in the molecular mechanisms that regulate these processes, knowledge has been rapidly expanding using reductionist models of cell culture of inflammation and epithelial and endothelial barrier function.

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-alpha) is involved in the pathogenesis of intestinal disorders and is found at increased levels in patients with cystic fibrosis (VAN DEVENTER, 1999; SALVA, et al., 1996). Using in vitro model systems, TNF-alpha has been shown to directly affect the function of TJs in various epithelial and endothelial cell lines. However, there are divergent reports on this statement, which complicates the interpretation of TNF-alpha. These may reflect specific variation of cell type, as well as differences in length and dose of cytokine treatment (MARANO et al., 1993; GRANT-TSCHUDY; WIRA, 2005).

Interferon gamma (IFN-gamma) is a Th1 pro-inflammatory cytokine found at elevated levels in the intestinal mucosa of patients with inflammatory bowel diseases. In addition to its immunomodulatory role during inflammation, IFN-γ acts to modify epithelial and endothelial barrier function (NAKAMURA et al, 1992; BOUMA; STROBER, 2003). There is a divergence in the functionality of IFN-gamma dependent on the place of operation. For example, in the inflammation model cell culture systems, direct treatment with IFN-gamma increases the paracellular permeability of endothelial and epithelial monolayers, already in epithelial cells of the airways, exposure to IFN-gamma has anti-inflammatory properties and promotes epithelial barrier function (COHN; ELIAS; CHUPP, 2004).

The combination of treatment with TNF-alpha and IFN-gamma results in localization errors in the cytoplasm of proteins das TJs, such as JAM-A, claudin 4 and claudin 5 (OZAKI et al., 1999; PRASAD et al., 2005). The myosin light chain (MLC) phosphorylation increases with the combined treatment of TNF-alpha and IFN-gamma in Caco-2 epithelial cells

(ZOLOTAREVSKY et al., 2002). The involvement of motor myosin is essential for permeability changes induced by IFN-gamma associated with TNF-alpha in T84 cells, since inhibition of myosin with pharmacological inhibitors reduces endocytosis and cytokine-treated cells (ZOLOTAREVSKY et al., 2002). Although it is not necessary for the internalization of the protein in T84 cells, myosin light chain kinase (MLCK) is positively regulated in Caco-2 cells treated with IFN-gamma and TNF-alpha (UTECH et al., 2005; ZOLOTAREVSKY et al., 2002). The mechanism of synergy of IFN-gamma and TNF-alpha still needs to be further studied, however it is believed that IFN-gamma stimulates cultures for treatment with TNF-alpha positively regulating the TNF-alpha cell surface receptor (WANG et al., 2006).

Finally, a large group of cytokines that influence intestinal permeability are interleukin (IL) as, which include IL-1, 4, 6, 8, 10 and 13.

Interleukin-1 is a pro-inflammatory cytokine type 1 that is elevated in the intestinal mucosa of patients with intestinal disorders (LIGUMSKY et al., 1990; BROIDE et al., 1992). By testing in both cell culture systems, epithelial and endothelial in vitro, the addition of IL-1 to growth media increased paracellular permeability to ions and small molecules (MARCUS et al., 1996; AL-SADI; MA, 2007). In these tests with Caco-2, treatment with IL-1 resulted in decreased levels of occludin protein, at least in part, due to reduced levels of occludin mRNA (AL-SADI; MA, 2007). This is consistent with previous discoveries in astrocytes, in which treatment with IL-1β suppressed occludin protein levels (DUFFY et al., 2000). These studies clearly highlight a relationship between the abnormal expression of occludin and the permeability of TJs.

Treatment with IL-4 increases permeability in the intestinal epithelial T34 cell model, as well as in the epithelial cells of the Calu-3 airways, which after 24 hours of treatment, demonstrate the decrease in TER (AHDIEH; VANDENBOS; YOUAKIM, 2001; COLGAN et al., 1994). The small flow of molecules through the epithelium increases with prolonged treatment with IL-4 (48 h), which corresponds to a decrease in the protein levels of ZO-1 and occludin (AHDIEH; VANDENBOS; YOUAKIM, 2001). An interesting finding relates the addition of intestinal epithelial permeability induced by IL-4 to the increase in the expression of claudine protein type 2 (PRASAD et al., 2005; WISNER et al., 2008). Because pore formation is related, the increase in the expression of claudine 2 alters cell permeability, as exemplified by the overexpression of claudine 2 in epithelial cells, which decreases

transepithelial resistance (TER) and confers relative increasing in the passage of Na+ (VAN ITALLIE; FANNING; ANDERSON, 2003).

Mice knockout for IL-6, increased intestinal permeability for small molecules has been associated with zo-1 stability in TJs (YANG et al., 2003). Consistent with this, treatment with IL-6 in vitro also increases permeability through endothelial cells and induces the displacement of ZO-1, the remodeling of the actin structure and the increase in actin contractility (DESAI et al., 2002).

IL-10 differs in the performance on permeability, because it opposes the influence of pro-inflammatory cytokines, such as IFN-gamma among others (MADSEN et al., 1997; OSHIMA et al., 2001). Mazzon et al. (2002) observed that mice knockout to IL-10, in a spontaneous colitis model, have increased levels of pro-inflammatory cytokines TNF-alpha, IL-1 and IL-6. Direct treatment of airway epithelial cells with IL-13 causes decreased TER and increased manitol flow and lower levels of ZO-1 protein (AHDIEH; VANDENBOS; YOUAKIM, 2001). IL-13-mediated barrier dysfunction in T84 cells also correlates with increased levels of claudine protein 2 (PRASAD et al., 2005). IL-13 and IL-4 act synergistically to stimulate the classical via STAT6, although the involvement of this pathway in the structure and function of the TJs has not been directly evaluated (HELLER et al., 2004).

2.2.3 The close relationship between microbiota and intestinal immunity

Early influence of the microbiota in the formation of the immune system

With the beginning of the use of germ-free animals (GF), the concept emerged that the microbiota influences the immune system. In the first studies where the authors compared GF rodents and microbiota-colonized animals, they observed highly significant and frightening physiological differences. Including an increased cecum, mainly due to the accumulation of undegraded mucus and reduced gastrointestinal motility (GUSTAFSSON; MIDTVEDT; STRANDBERG, 1970), since the resident microbiota assists in digestive functions, and in the absence of it there are losses of these functions. Along with this, the morphology of IECs in GF animals is completely altered, which includes longer villi and shorter crypts compared to those observed in common mice (ABRAMS; BAUER; SPRINZ,1963) and with reduced antimicrobial peptides (VAISHNAVA et al., 2008).

It is noteworthy that the absence of coendous bacteria profoundly influences the structural and functional development of the immune system, such as defects in the development of lymphoid tissue within the spleen, thymus, and lymph node (BAUER et al.,

1963; GORDON et al., 1966). In addition, these authors reported that these structural abnormalities were more remarkable near the mucosal interface, suggesting that interactions with specific communities of microorganisms directly modulate the development of intestine-associated lymphoid tissue (GALT). For example, ILFs are minimally present in the absence of microbiota in the small intestine, but not in the colon (MOSCONI et al., 2013; BAPTISTA et al.,2013). These studies make it clear that the microbiota participates in the maturation of the immune system and that the colonization process, affected by several factors, may be important in the development of a normal immune system in a healthy individual.

During the first years of life, the process of colonization of mucous surfaces is characterized by fluctuating changes in microbial diversity, progressing to a relatively stable equilibrium point throughout adulthood, except in the occurrence of environmental disturbances (SPOR; KOREN; LEY, 2011). In addition, bacterial colonization of the mucosa at the beginning of life occurs concomitantly with the development, expansion, and education of the immune system of the mucosa, this fact can directly and/or indirectly affect the immune profile of the individual (GEUKING et al., 2011; RAKOFF-NAHOUM; MEDZHITOV, 2008).

Influence of the immune system of the intestinal mucosa on the intestinal microbiota

With the technological advance in microbiota analyses, information emerged on the numerous factors that determine the composition of the microbiota. Among them, how the formation of the structure of microbial communities associated with the host is affected by nutrients, whether derived from the diet or even from endogenous sources of the host (SONNENBURG et al., 2005; SONNENBURG et al., 2010). Evidence in the literature also suggests that the immune system is an important contributor to the control of macrobiotic composition.

Certain antibacterial proteins secreted by epithelial cells can shape the composition of intestinal microbial communities. An example mentioned earlier and that has this potential, are α-defensin, small antibacterial peptides secreted by Paneth cells of the epithelium of the small intestine (ZASLOFF, 2002). Salzman et al. (2003) when analyzing the microbiota in mice that were deficient in functional α-defensin or that human α-definin-5 was overexpressed, showed that, although there was no impact on the total number of colonizing bacteria, there were substantial changes dependent on α-dessins in the composition of the microbial community in the two strains of mice. However, it is not known how far these

secreted immune agents can affect luminal microorganisms. For example, the impact of α-desinsin-5 on the composition of the luminal community contrasts with REGIIIg antibacterial lectin, which limits the penetration of bacteria into the epithelial surface but does not alter the luminal communities (VAISHNAVA et al., 2011). With this we can infer that some antimicrobial peptides, such as α-defensin, have luminal range and can modify the general composition of the microbiota, while others, such as REGIIIg, have restricted local effects.

The impact of the immune system on the composition of the microbiota is observed in a series of immune deficiencies that alter microbial communities in ways that predispose to the disease. Corroborating this statement, Garrett et al. (2007), studied mice that do not have the transcription factor T-bet (encoded by Tbx21), which governs inflammatory responses in cells of the innate and adaptive immune system. When crossing Tbx21-/- mice with Rag2-/- mice, which do not have adaptive immunity, the progeny Tbx21-/-/Rag2-/- developed ulcerative colitis in a microbiota-dependent manner. It is concluded that the altered microbiota was sufficient to induce the disease, thus being considered dysbiotic. Similarly, mice without the TLR5 flagellin receptor exhibit a syndrome involving insulin resistance, hyperlipidemia and increased fat deposition associated with changes in microbiota composition (VIJAY-KUMAR et al., 2010).

A third example of immune system-induced dysbiosis is shown in mice deficient in the expression of epithelial cells of the inflammasome component NLRP6. This deficiency induced the development of an altered microbiota, with prevalence of members of the phylum *Bacteroidetes*, associated with increased recruitment of inflammatory bowel cells and susceptibility to chemically induced colitis (ELINAV et al., 2011). Once again it is evident that only the dysbiosis process is sufficient to direct intestinal inflammation, wild mice conventionally created that acquire the biotic microbiota show similar immunopathology (ELINAV et al., 2011). These findings suggest that the immune system exerts some control over the microbial composition of the host.

It is not yet known exactly what extent the influence that the immune system exerts on the resident microbiota, although it is evident that it affects the composition of the community at the species level. But it is not yet known whether this effect can be extrapolated to other organic systems, such as the respiratory tract, urogenital tract, and skin.

Influence of intestinal microbiota on the immune system of the intestinal mucosa

The challenges to understand the numerous interactive pathways between the immune system and the host are closely linked to the general health, not only of humans, but also of animals. This is also where this has increased considerably in studies that can help answer the numerous questions that involve this mutual interaction.

IECs can recognize the microbiota through the TLR-MyD88 signaling pathway, resulting in several responses that are critical to maintaining the host's microbial homeostasis. Bacterial signs dependent on MyD88 are also necessary for the induction, by LPS or flagellin, of epithelial antimicrobial proteins, such as REGIIIγ (VAISHNAVA et al., 2011). Flagellin signals are relayed through TLR5 expressed by CD103+ CD11b+ dendritic cells in the lamina itself, stimulating the production of IL-23, which, in turn, promotes the expression of IL-22 by innate lymphoid cells (KINNEBREW et al., 2012). IL-22 then stimulates the production of REGIIIg, which is also secreted after direct activation of MyD88 in epithelial cells (VAISHNAVA et al., 2011). Clearly this example evidences the importance of commensals in inducing innate host responses but represents a tiny fraction of the microbiota's range of effects on the host immune system.

The individual commensal species influence the composition of the subsets of T lymphocytes of the lamina itself that have distinct effector functions. Homeostasis in the intestinal mucosa is maintained by a system of checks and balances between potentially pro-inflammatory cells, which include TH1 cells that produce IFN-gamma; Th17 cells that produce IL-17a, IL-17f and IL-22; several innate lymphoid cells with cytokine effector characteristics that resemble Th2 and Th17 cells; and foxp3+ anti-inflammatory regulatory T cells (Tregs) (GABORIAU-ROUTHIAU et al., 2009). Colonization of mice with segmented filamentous bacteria (FBS) results in the accumulation of Th17 cells and, in smaller importance, an increase in Th1 cells (IVANOV et al 2009).

The response, in the face of the commensal microorganisms, by regulatory T cells differs according to the models used to study inflammation. Mouses that have been subjected to chemical damage or pathogen-induced mucosa, the response of Th17 cells is beneficial and promotes healing. In contrast, Th1 and Th17 cells, as well as IL-23-dependent innate lymphoid cells, promote colitis in models in which Treg cells are inactivated (ABRAHAM; CHO, 2009). Possibly inflammatory bowel diseases in humans are triggered by imbalance of lymphoid cell subgroups in response to commensal microbiota. Supporting this information, other studies report the strong link of IL23R polymorphisms with Crohn's disease, a severe condition with intestinal inflammation, and severe enterocolitis associated with IL10 and

IL10R mutations (GLOCKER et al., 2009). It has long been suggested that systemic autoimmune diseases have links to infections, but solid confirmations are still lacking. To prove this information, some of them were based on animal models, they reinforced the notion that the microbiota commensal contributes to systemic autoimmune and allergic diseases in distal sites to the intestinal mucosa. Several models of mice for autoimmunity depend on colonization status. In all these models, the induction of TH17 cells in the intestine has a profound influence on systemic disease (LEE et al., 2011).

Many essential details of the immune response to the microbiota need to be discovered, it is not yet known how the aper microorganisms influence the differentiation of immune cells, such as TH17 and Treg cells, or which microbial molecules drive the differentiation of these cells. In fact, understanding these factors will bring highly positive responses to human and animal health.

2.3 Strategies

2.3.1 Organic acids

Generally speaking, organic acids act in the same way. They dissociate in aqueous media, where the pKa must be equal to or less than the pH of the medium, releasing H+ ions and therefore acidifies the solution (CANIBE et al., 2001). Remembering that at pH higher than pKa there is still dissociation, but in lower intensity.

The low stomach pH is critical to maximize the activity of the pepsin enzyme (DESAI; RANADE, 2007), produced through the activation reaction of pepsinogen, which is H+ dependent. Piglets in the post-weaning period have low activity of this enzyme due to HCl production and reduction in lactic acid production (SUIRYANRAYNA; RAMANA, 2015). Some of these effects are associated with low consumption in this period. Along with this, another factor that hinders the reduction of pH is the buffering effect of diets, which is characterized by the ability to resist changes in pH (VIOLA; VIEIRA, 2003). Therefore, the use of organic acids in the diets, mainly of weaned piglets, can be a component that favors the reduction of the pH of the digesta due to its mode of action, thus improving the digestibility of nutrients by the effect of increasing the activity of digestive enzymes such as pepsin and trypsin (LONG et al., 2018). In addition to indirect effects, organic acids act directly on bacteria as bacteriostatic and bactericides. In their non-dissociated form, they are lipophilic so they can cross the cell membrane of Gram-negative bacteria. Once inside the cell, the higher

cytosolic pH causes the acid to dissolve, releasing hydrogen ions, which consequently reduces intracellular pH (KOOPMANS et al., 2016). Microbial metabolism is dependent on enzyme activity, which is depressed at lower pH. To correct the balance, the cell is forced to use energy to expel protons through the membrane through the H+ATPase pump to restore cytoplasmic pH. During a period of exposure to an organic acid, this may be enough to prevent multiplication and even kill the bacterial cell (LUCKSTADT; MELLOR, 2010). In addition to predicting the growth of intestinal microbial pathogens, it favors non-pH-sensitive bacteria such as *Lactobacilli* and *Bifidibacterium* (LONG et al., 2018). These effects influenced the increase in bacterial diversity and richness (LI et al., 2018). However, each acid has minimum inhibition concentrations that are specific (STRAUSS; HAYLER, 2001).

Some mechanisms inherent to the action of organic acids are still unknown and arouse interest due to the importance of some results. In the study by Diao et al. (2016), benzoic acid increased the activities of SOD and GSH-PX in the jejunal mucosa of pigs, which could increase antioxidant capacity, and then improve jejunum morphology. However, there have been no other reports related to the effects of benzoic acid on antioxidant capacity in animals, and the mechanism remains uncertain, so more research is clearly needed. Also in the same study, benzoic acid increased the concentration of GLP-2 and the relative gene expression in the jejunal mucosa, which found that benzoic acid could improve the morphology of the jejunum via positive regulation of GLP-2 in the swine intestine.

It has been reported that a combination of organic acids would increase the effectiveness of acidification due to its dissociation capacity over a wide range of pH values, thus maintaining optimal pH through the intestinal tract (RAVINDRAN; KORNEGAY, 1993). However, the efficacy of the use of organic acids in pigs varies with the types and combinations of acid, the health status of the animal and the characteristics of the diet, particularly the buffering capacity of the diet (BLANK et al., 1999; MROZ et al., 2006).

Supplementation of a single acidifier, such as formic acid or sorbic acid, improved growth rate and feed efficiency, as reported by Partanen and Mroz (1999) and Overland et al. (2008). Similarly, Walsh et al. (2007) indicated that the addition of 0.4% organic acid mixtures, such as fumaric, lactic, propionic, citric, and benzoic acid, improved animal performance. Li et al. (2008) reported that 0.5% of organic acid mixtures such as butyric acid, fumaric and benzoic in the diet of weaned piglets improved growth performance. However, some results indicate absence or negative responses with the use of a single acidifier in the diet, such as fumaric, citric or formic acid (MANZANILLA et al., 2004) or in cases of some

mixtures such as formic acid, lactic acid, and volatile fatty acids (LEE et al., 2007). Other reports with different types of organic acids indicated that the inclusion of organic acid as 2% benzoic acid (KLUGE et al., 2010) in the diet of lactating sows improved nutrient digestibility, 0.5% phenylacetic acid (WANG et al., 2009a) improved nutrient digestibility in weaned piglets and egg production trends in laying hens (WANG et al., 2009b) and Franco et al. (2005) also reported that the combination of lactic acid with formic or fumaric acids numerically increased the digestibility of DM in weaned piglets. However, Kil et al. (2006) indicated that there was no positive response in nutrient digestibility with the inclusion of lactic, formic or fumaric acids in weaned piglets.

Just as the addition of 1.5% citric acid in the diets did not significantly affect the pH, the concentration of volatile or nonvolatile fatty acids, or microflora (total anaerobic, *Lactobacillus, Clostridia, E. coli*) in the content of the stomach, jejunum, cecum, or lower colon diet of weaned piglets (RISLEY et al., 1991; 1993). In addition, it did not affect the incidence or score of diarrheas after a challenge with *E.coli* in the post-weaning period (RISLEY et al., 1993). In contrast to these results, Narayanan et al. (2008) concluded that supplementation with 2% citric acid in the initial diet of piglets before weaning had a positive effect on the reduction of *E.coli* count in fecal content, with improved weight gain and reduction of enteropathogenic mortality until weaning. Citric acid has a moderate antimicrobial action, since it can be metabolized by the bacterial cell itself, but when entering the Krebs cycle, it can also serve as an energy source for the host (GRILLI et al., 2015). On the other hand, when associated with sorbic acid and timol (25% citric acid, 16.7% sorbic acid and 1.7% of timol – microencapsulated) the blend induced a faster maturation of the intestinal mucosa, decreasing local and systemic inflammatory pressure, resulting in a less permeable intestine and, eventually, improving the growth of prematurely weaned piglets (GRILLI et al., 2015). In a recent study Walia et al. (2017) using an encapsulated mixture of formic acid, citric acid, and essential oils, at 4 kg/ton of feed, in pigs 28 days pre-slaughter, reported a potential of the blend in preventing the increase of salmonella shedding. However, supplementation did not influence intestinal transport, nor did it reduce seroprevalence below the cutoff used for the high-risk category for *Salmonella* in Ireland (50%). Furthermore, it did not improve growth performance and, in fact, increased the feed cost per kg of live weight gain during the test. The longer duration of dietary supplementation can be justified, although the cost-effectiveness of this must be determined.

In order to validate the efficiency of a blend of organic acids with medium fatty acids, Kuang et al. (2015) tested the replacement of this blend by zinc oxide (ZnO) in the diet of weaned piglets. They concluded that supplementation contributed more than ZnO to the growth of piglets weaned in commercial conditions. They associated the benefit with increased digestibility of amino acids and immunity of piglets. Another finding was the increased proliferation of Lactobacillus after the consumption of organic acids, possibly due to increased expression of regulatory cytokines and a reduction in the expression of pro-inflammatory cytokines. In fact, there is divergence regarding the use of organic acids, which indicates that we still need more research. However, some acids are better known due to their wide use in swine diets and have been promoting encouraging results.

The benzoic acid is a good example. This acid has a relatively high ionization constant (pKa = 4.21), so it is found in the stomach in the non-dissociated form. With this it can cross the bacterial membrane and to find a pH greater than its pKa quickly dissociates, and can kill the bacteria (MELLOR, 2000). Benzoic acid and sodium benzoate are widely used as food conservatives, because in the non-dissociated form they act on fungi (KUNG; STOKES; LIN, 2003). According to Mroz (2005), benzoic acid increases the digestibility of amino acids and nitrogen, increases urinary acidity, and reduces ammonia emissions by waste. There are numerous beneficial effects that benzoic acid has shown. Several authors report a positive effect on the control of the intestinal microbiota, on the improvement of intestinal morphology and effects on nutrient digestibility (ALVARADO et al., 2013; DIAO et al., 2013, 2016; GHELER et al., 2009; GRÄBER et al., 2012; PAPATSIROS et al., 2011). Other authors have also observed improvements such as increased feed intake and weight gain of animals, greater degree of biodiversity of the gastrointestinal microbiota (TORRALLARDONA et al., 2007), improvement of total ileal digestibility of energy and nitrogen and a reduction in the amount of *E. coli* in piglet cecum (GUGGENBUHL et al., 2007). Silveira et al. (2018) observed better results for performance in the nursery phase, with its effects extended until termination. Diao et al. (2016) found a reduction in crypt depth and increased villous/crypt ratio in young piglet jejunum, with reflexes in nutrient digestibility and animal performance. In addition, it increased GLP-2 concentration and relative gene expression in the jejunal mucosa, as well as increased SOD and GSH-PX activities in the jejunal mucosa.

In order to potentiate the effects of benzoic acid, research was conducted with its association with essential oils. Essential oils have some effects similar to benzoic acid, except

that they have the ability to disrupt the cellular seem of bacteria that can result in lysis and leakage of macromolecules (LAMBERT et al., 2001; OUSSALAH; CAILLET; LACROIX, 2006). Therefore, since essential oils act on the cell wall of the bacterium, leaving it more permeable the acid can enter more easily to perform its dissociation inside the cell (VONDRUSKOVA et al., 2010). Huang et al. (2010) evaluated a high inclusion of essential oils compared to a mixture with benzoic acid, observed that piglets that received the inclusion of 1g/kg of essential oils had weight gain equal to those that received basal feed and animals that received the inclusion of 1g/kg in association with 3g/kg of benzoic acid increased weight gain.

Another promising organic acid is butyrate. In the small intestine, butyrate increases proliferation, differentiation and maturation and reduces apoptosis of normal enterocytes, mediated by its influence on gene expression and protein synthesis (SENGUPTA; MUIR; GIBSON, 2006). In the small intestine, the depth of the crypt and the height of villi increase largely in piglets weaned with butyrate (MANZANILLA et al., 2006). Butyrate has been shown to exert trophic effect on ileal and jejunal epithelial cells, obviously by indirect pathways, through a neurohormonal mechanism (FRANKEL et al., 1994). More recent molecular studies investigating cell proliferation, damage, and programmed death, mainly apoptosis, revealed that butyrate accelerates the maturation of the intestinal mucosa during development or repair after injury (KOTUNIA et al., 2004). In rodents and pigs, all studies showed that the weight of the large intestine is higher in animals fed a butyrate-producing diet, compared with controls (LACORN; GOERKE; CLAUS, 2010). Increased concentrations of butyrate are associated with morphological changes in mucosa (MENTSCHEL; CLAUS, 2003). In the absence of butyrate, there is a sudden increase in apoptosis in colonocytes (LUCIANO et al., 2002).

In a large study conducted by LONG et al. (2018), which aimed to test blends of organic acids as antibiotic substitutes, they achieved promising results. Four diets were tested: basal diet (DB), Diet with growth promoters- ATB (DB + promoters), blend 1- B1 (Ac. Formic, ac. Acetic and ac. Propionic) and blend 2- B2 (Phenolic compounds, Butyrate, Ac. Sorbic, ac medium chain fatty and essential oils). As results of the research, it was demonstrated that the overall performance of growing pigs was improved with the use of blends when compared to DB and were like pigs fed ATB diet. The results may be due to improvement in apparent digestibility of dry matter, ether extract, total carbohydrates, FDN, FDA, phosphorus. There was an increase in villi height and in the relationship between villus

height and crypt depth in jejunum and ileum for blends compared to DB diet. In addition, the lower content of E. coli in feces and the health status measured by decreased diarrhea score and increased immunological indices, along with antioxidant indices. The results suggest that the blends can be used to replace antibiotics growth promoters, with positive effects on performance, serum immunity, intestinal morphology, and microbiota in weaned piglets.

However, the mechanism of action of organic acids is unclear (GUILLOTEAU et al., 2010), possibly its effects may be due to the different forms of individual action in different sites of the gastrointestinal tract (FRANCO et al., 2005; KASPROWICZ-POTOCKA et al., 2009). The great inherent limitation of the effective dose of organic acids, where it can express its maximum potential, is around the rapid absorption, metabolism, or both, which suffer when entering the duodenum. This could be overcome by the microencapsulation of active compounds in a matrix that could dissolve as it passes through the intestine (PIVA et al., 2007). For this reason, microencapsulation could potentiate the effects of organic acids (CHO; SONG; KIM, 2014). Microencapsulation can be used in a wide range of applications, from slowing the absorption of drugs (PIVA et al., 1997) and protecting amino acids and proteins from ruminal degradation (NOEL, 2000) to providing technological advantages in the handling of irritating or corrosive substances. In the study by Piva et al. (2007), they concluded the microencapsulate promote gradual release of AO and allows its action in the different compartments of the gastrointestinal tract, potentiating its mode of action.

However, we must pay attention to an important aspect that also potentiates the individual effect of each organic acid, which is the blend of acids. In fact, microencapsulation is critical to extend the effect of organic acid along the gastrointestinal tract and we can use this technology to encapsulate a blend of organic acids with different pKa's and functionalities. It has been reported that a combination of organic acids would increase the effectiveness of acidification due to its dissociation capacity over a wide range of pH values, thus maintaining optimal pH through the intestinal tract (RAVINDRAN; KORNEGAY, 1993).

2.3.2 Polyphenols

Polyphenols have a common chemical structure, derived from benzene, attached to a hydrophilic group. Based on their structure and the way in which the polyphenolic rings are linked together, they are classified into four families: phenolic acids, lignans, stilbenes (resveratrol) and flavonoids or bioflavonoids (flavones, flavanones, catechins and

anthocyanins) (MARTINS; NICOLETTI, 2016). This last group is the largest and most studied, having more than 5,000 identified compounds, and its main source is food, fruits, vegetables, teas, cocoa, soy, among others.

The main biological activities attributed to flavonoids are antioxidant action and inhibition of enzymatic activities. These aspects justify the fact that they suppress inflammation, in vivo and in vitro, reducing the severity of different inflammatory diseases (PEREZ-CANO et al., 2014). In the study by Galsanov, Turova and Klimenko (1976) for the first time described the anti-inflammatory activity of quercitrin, when administered at doses of 25 and 100 mg/kg, in a rat model of allergic intestinal inflammation. Since then, many studies describing the impact of flavonoids in various experimental models of rodent colitis have been published. These studies revealed the anti-inflammatory activity of different intestinal flavonoids, including both glycosides and aglycones, and those belonging to different chemical classes, such as flavonols (quercetin, quercitrin, rutin), flavanones (naringenin), flavones (baicalin, chrysin), catechins (epigallocatechin-3-gallate (EGCG)), isoflavones (genistein, daidzein, glabridin), anthocyanidins (cyanidin-3-glucoside (C3G)) and chalcones (cardamonin) (VEZZA et al., 2016). Currently, it is difficult to establish a relationship between flavonoid type and activity, as the number of flavonoids tested so far is low. However, of all the flavonoids tested, quercitrin was found to be the most potent, presenting preventive or curative properties at doses of 1 and 5 mg/kg (SANCHEZ DE MEDINA et al., 1996). While the dosage of other flavonoids is wide, ranging from 10 to 25 mg/kg when glycosides are considered, and between 10 and 200 mg/kg in the case of aglycones (VEZZA et al., 2016).

It is not yet fully understood whether the effects of flavonoids are direct on the inflamed colonic tissue or whether they are systemic after their absorption and subsequent metabolism, or both. An example is the flavonoid quercetin, the oral administration of its aglycone at doses of 9 mg/kg has no effect (CASTANGIA et al., 2015), however its glycoside derivative, quercetin, has intestinal anti-inflammatory effects in multiple models. of colitis with dosage below 5 mg/kg. Aglycones are absorbed in the small intestine while most of the glycosides reach the colon, where they are cleaved by the microbiota and the aglycone portion is released (SANCHEZ DE MEDINA et al., 1996). This could explain why glycosidic derivatives could be more potent in colonic inflammation when administered orally (COMALADA et al., 2005).

The alteration of the immune response during inflammation is associated with an increase in the release of pro-inflammatory cytokines, including IFN-gamma, TNF, IL-6, IL-1β, chemokines such as IL-8 among other factors. Flavonoids have been found to regulate the altered immune response that occurs in intestinal inflammation. Several studies report that the administration of flavonoids notably decreased the increase in the levels of different cytokines evaluated in the inflamed colon (REN et al., 2015; SHIN et al., 2009; BRUCKNER et al., 2012; OZ; CHEN; DE VILLIERS, 2013). T cells are important actors in the development of inflammatory bowel diseases (YAMAJI et al., 2012). These diseases have been associated with an increased percentage of Th1 and Th17 cells in the mesenteric lymph nodes, which correlates with the overexpression of pro-inflammatory cytokines such as IFNγ, IL-17A and IL-17F (VEZZA et al., 2016). The beneficial effects observed with flavonoid derivatives in induced colitis in mice were related to a downregulation of the proportion of both Th1 and Th17 cells and, therefore, a reduction in the release of cytokines by these cell subtypes in the colonic tissue (TAO et al., 2013). In *in vitro* experiments, incubation of LPS-activated macrophages with quercetin resulted in reduced levels of IL-1β and TNFα when compared to cells stimulated without flavonoid treatment (COMALADA et al., 2005; CUI et al., 2014). In addition, quercetin exerts antiproliferative effects, reducing the production of IFN-gamma and TNF-alpha in T lymphocytes (SERRA et al., 2013).

Other studies have reported that different flavonoids such as quercetin (SANCHEZ DE MEDINA et al., 1996), rutin (GALVEZ et al., 1997), hesperidin (CRESPO et al., 1999) and morin (OCETE et al., 1998) have been reported improved absorptive function and intestinal barrier integrity leading to fewer symptoms of diarrhea, which are frequent in intestinal inflammation. These effects were related to the ability of flavonoids to inhibit muscle contractility, increase intestinal motility, and reduce intraluminal accumulation of intestinal fluid, as evidenced in different experimental studies (DI CARLO et al., 1993; GALVEZ et al., 1996; MELI et al., 1990). Azuma et al. (2013) also reported that flavonoid treatment resulted in an improvement in the permeability of the epithelial barrier, by preserving the function and structure of tight junctions. In addition to a direct effect on tight junctions, indirect mechanisms may also explain the beneficial effects of flavonoids in preserving intestinal barrier function. As stated earlier, the fact that flavonoids regulate pro-inflammatory cytokines, such as IFN-gamma, TNF or IL-6, indirectly guarantees the protection of barrier functions, as these directly interfere with these functions by mechanisms independent of (SUZUKI; YOSHINAGA; TANABE, 2011).

Most of the flavonoids tested were able to improve oxidative stress, through the reduction of lipid peroxidation, together with an improvement in different antioxidant markers, including sulfhydryl-derived compounds, or an increase in different enzymatic activities with antioxidant properties (AL-REJAIE et al., 2013), such as GSH, SOD and GPO and reducing tissue malondialdehyde (MDA) levels (BRUEWER et al., 2003; SUZUKI; YOSHINAGA; TANABE, 2011).

Prevention of free radical damage caused by flavonoids can occur in several ways. One of them is the direct elimination of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable and less reactive radical, so flavonoids stabilize the reactive oxygen species, reacting with the reactive compound of the radical, and because of their high reactivity in the hydroxyl group, the radicals are inactivated (PANCHE; DIWAN; CHANDRA, 2016). Korkina and Afanasev (1997) found that flavonoids such as epicatechin and rutin are powerful radical scavengers and that rutin's scavenging capacity may be due to its inhibitory activity on the xanthine oxidase enzyme. Kerry and Abbey (1997) reported that, by eliminating free radicals, flavonoids can inhibit LDL oxidation, protecting them, and theoretically having a preventive action against atherosclerosis.

Different studies have reported that diets containing bioactive compounds, such as phenolic compounds and tannins, can be considered as possible complementary treatments for intestinal diseases due to their antimicrobial and antioxidant capacity (PARK et al., 2005). In line with this statement, it was proposed that the impact of naringenin on the composition of the microbiota may also contribute to the beneficial effects exerted by this compound on intestinal inflammation, given its inhibitory effect on the growth and adhesion of *Salmonella typhimurium* in Caco cell cultures. -2 human (PARKAR; STEVENSON; SKINNER, 2008). In contrast, the same study revealed that this flavanone enhanced the proliferation and adhesion of the probiotic *L. rhamnosus*, known to promote beneficial effects on human intestinal inflammation (GHOURI et al., 2014). Furthermore, the antimicrobial effect of flavonoids and ability to stop bacterial growth has also been reported, which can also have a positive impact on intestinal inflammation (STEINMANN et al.2013).

Interestingly, although further studies are needed, an effect of flavonoids on the β-ketoacyl acyl transporter protein synthase III (KAS III), which initiates fatty acid synthesis in bacteria, has been reported to be a key target enzyme to overcome the antibiotic resistance problem. Lee et al. (2011), while working on known inhibitors of flavonoids β-KAS III

against methicillin-resistant bacteria, found that flavonoids such as naringenin and eriodyctiol are potent antimicrobial inhibitors of *Staphylococcus aureus* KAS III.

The behavior of other polyphenols is very similar to flavonoids, many of the mechanisms of action are identical, but we still observe particularities between the compounds. Similar to other polyphenols, resveratrol has high absorption, low bioavailability, and rapid clearance from the bloodstream (WALLE, 2011). The mechanisms by which resveratrol acts are similar to other polyphenols, based on in vitro studies, it can inhibit cell proliferation (VANAMALA et al., 2010), induce apoptosis (WANG et al., 2011) and block the progression of the disease. cell cycle (FILIPPI-CHIELA et al., 2011) in various types of cancer (WALLE et al., 2004). In vitro antioxidant assays have shown that resveratrol has a greater ability to scavenge free radicals than vitamin C and vitamin E (GÜLÇIN, 2010). Like flavonoids, resveratrol can also inhibit TNF, IL-1β, nitric oxide and NF-kB in peritoneal macrophages (MA et al., 2006) and shows a dose-dependent antiestrogenic activity similar to quercetin (BRGLEZ MOJZER et al., 2006). Sharma et al. (2007) reported that resveratrol inhibits the release of pro-inflammatory cytokines by peritoneal macrophages and stimulates the expression of IL-10 (SHARMA et al., 2007), as well as iNOS (RENAUD; MARTINOLI, 2017).

The lignans have been associated with several health properties, such as protection against LDL oxidation and inhibition of cancer cell growth in skin, breast, prostate, colon, and lung tissues (HIRANO et al., 1990; KARDONO et al., 1990). Indeed, clinical trials have reported a reduction in the risk of postmenopausal breast cancer, specifically the ER+/PR+ subtype, in women with high consumption of plant lignans (TOUILLAUD et al., 2007) and a reduction in breast tumor growth. in patients who consume flaxseed daily (THOMPSON et al., 2005). Regarding its potential as a free radical scavenger, the results differ. Li et al. (2010) found no antioxidant activity for this compound. In contrast, a recent study detected remarkable free radical scavenging activity (KOCH et al., 2015). Kang and Wang (2010) and Yi et al. (2011) also observed significant antioxidant activity, but IC50 values varied considerably, between 8.6 μM (KANG; WANG, 2010) and 153.46 μM (YI et al., 2011).

Several authors have carried out studies to verify the antioxidant potential of phenolic acids, with the aim of replacing synthetic antioxidants (DURÁN; PADILLA, 1993). The greatest antioxidant potential found for phenolic acids is when two hydroxyl groups are at positions 3 and 4, therefore, the antioxidant activity is in the following order: caffeic acid > 3,4-dihydroxybenzoic acid > synapic > syringic > ferulic > p-coumaric > vanillic

(MARINOVA; YANISHLIEVA, 1992). The antioxidant potential of caffeic and ferulic acids was also investigated in vitro, using rat liver microsomes (PULLA REDDY; LOKESH, 1992). However, the results were significant only for caffeic acid. Other effects inherent to phenolic acids are very similar to those found in flavonoids.

2.3.3 Tryptophan

The amino acids are considered as the main energy substrates in the intestinal mucosa, being limiting constituents of intestinal barrier proteins and responsible for acting in regulatory processes of immune responses and oxidative stress (WU, 2013). Weaning is believed to reshape amino acid metabolism, providing an increase in the need for these components (CHALVON-DEMERSAY et al., 2021).

Tryptophan is classified as an essential amino acid for pigs, being mainly derived from the diet (KALUZNA-CZAPLINSKA et al., 2019). It is also the main protein component of animals, presenting various physiological and biochemical functions during the processes of growth, immune regulation, digestion, protein synthesis and anti-stress effect. Thus, the addition of tryptophan in pig production can improve the performance and health of weaned piglets (MOU et al., 2019).

From the digestion of dietary proteins in the small intestine, tryptophan is released, which is absorbed by the intestinal absorptive cells, allowing it to reach the bloodstream and remain circulating, contributing to protein synthesis (YU et al., 2017; MU et al., 2017). This amino acid exerts modulatory functions at various levels of the gut-brain axis, being the only precursor of serotonin, which in turn acts in the modulation of central neurotransmission and participates in the regulation of enteric physiological mechanisms (GAO et al., 2020), and later. it acts in the synthesis of melatonin (O'MAHONY et al., 2015). Furthermore, tryptophan can be metabolized to kynurenine, tryptamine, and indole, modulating immune, neuroendocrine, and intestinal responses (GAO et al., 2020).

Studies have shown that tryptophan metabolism is markedly affected in various cell types, such as those of the immune system and neurons, in response to pro-inflammatory conditions in individuals undergoing stressful conditions (MIURA et al., 2008; YAO et al., 2011). Findings indicate that tryptophan may be an immunomodulatory agent for the treatment of intestinal irritation. This is since it contributes to the decrease in the expression of pro-inflammatory cytokines (TNF-alpha, interleukin 6, interferon-gamma, interleukin

12p40, interleukin 1β, interleukin 17 and interleukin 8) and intracellular adhesion molecule-1 (CONNIE et al., 2010).

There is a paucity of studies that correlate the specific impacts of certain amino acids on the microbiome and gut health as a whole (LIANG et al., 2018). As far as is known, tryptophan, through the different biologically active compounds that it is metabolized, regulates processes of inflammation, immune response, and neurological functions (YAO et al., 2011; CERVENKA et al., 2017), as well as having an intimate in relation to the abundance of bacteria present in the digestive tract. Evidence reveals that commensal gut bacteria regulate tryptophan availability and metabolism in this organ (GAO et al., 2020).

The protein intake can impact the microbiota to modulate gut structures and functions (LIANG et al., 2018). Commensal gut bacteria actively metabolize tryptophan, producing indole-3-acetic acid and indole, two metabolites that can improve intestinal mucosal barrier function, such as immune function (ZELANTE et al., 2013; JIN et al., 2014; VENKATESH et al., 2014; LAMAS et al., 2016; BARRATT et al., 2017). Concurrently, in vitro studies demonstrate that tryptophan increases the abundance of tight junction proteins and turnover of proteins in intestinal epithelial cells. (WANG et al., 2015). As well as its supply to weaned piglets is associated with behavioral changes in animals. There have been reports of a significant reduction in the frequency and duration of fights between piglets, resulting in improved intestinal integrity (KOOPMANS et al., 2006). Therefore, tryptophan is configured as a functional amino acid with a recognized impact on the physiology and metabolism of the host (WANG et al., 2015).

The serotonin (5-hydroxytryptamine) is a monoamine molecule which is synthesized from tryptophan, being responsible for a prominent role in the central nervous system. The serotonin is involved in the modulation of emotional control, consumption, sleep, and pain processing (ISRAELYAN; MARGOLIS, 2018). It is mainly produced from enterochromaffin cells, which are a specialized type of intestinal epithelial cells, justifying the fact that 90% of serotonin is in the gastrointestinal tract (MAWE; HOFFMAN, 2013). Even though there are differences in serotonin availability along the gut-brain axis, both sites are shown to be dependent on tryptophan hydroxylase (TPH). This is found in two isoforms: TPH1, expressed mainly in enterochromaffin cells, and TPH2, which is expressed locally in the brain (ISRAELYAN; MARGOLIS, 2018). TPH expressions can be influenced by many factors, suggesting its importance in terms of modulating serotonin availability and its functional consequences (GAO et al., 2020).

Crystalline amino acids (AA) are often used as a substitute for protein sources in swine and poultry diets. This allows a reduction in crude protein (CP) concentration and brings the formulations closer to the ideal protein concept, which brings environmental and economic benefits (MIRANDA et al., 2015). Reducing the concentration of CP with greater inclusion of crystalline AA in swine diets can result in increased nitrogen utilization, reduced post-weaning diarrhea and increased swine growth performance (STEIN; KIL, 2006; KIL; STEIN, 2010; WANG et al., 2018). The synthesis of crystalline AA occurs through the fermentation of bacteria in a culture medium containing sources of carbon, nitrogen, sulfur, and phosphorus (LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005). With advances in genome sequencing, most AA can be produced using mutant strains of Escherichia coli or Corynebacterium glutamicum (LEUCHTENBERGER; HUTHMACHER; DRAUZ, 2005). Once fermentation is complete, the first step in purification is the separation of AA from the fermented biomass (HERMANN, 2003). Due to this process, residual amounts of indispensable CP and AA are retained in the biomass, leaving a nutritionally rich by-product that is often not used (ALMEIDA; SULABO; STEIN, 2014). The use of biomass, before the extraction of specific AA, can be a viable option to replace sources of crystalline amino acids, as it is an opportunity to reduce manufacturing inputs and still provide a product rich in AA (HERMANN, 2003). The most common example of the use of biomass to replace crystalline AA is Lysine Sulfate which contains 54.6% of L-lysine with fermented biomass being commonly used in swine and poultry diets to replace Lysine HCl (SCHUTTE; PACK, 1994), with equivalent bioavailability (SMIRICKY-TJARDES et al., 2004; HTOO et al., 2016). Recently, fermented biomass products of AA including tryptophan have been developed for use in livestock and poultry diets. In the study by Smiricky-Tjardes et al. (2004) there was no difference in growth performance between pigs fed L-lysine (L-Lys) HCl and L-Lys sulfate with biomass, evidencing a similar relative bioavailability between the two sources of amino acids. Furthermore, when evaluating a source of tryptophan with biomass, WENSLEY et al. (2019) reported no evidence of a difference in performance with the same amino acid requirement using crystalline tryptophan. However, there is limited research available to determine its effectiveness as a source of AA for growing pigs and chickens.

3 FINAL CONSIDERATIONS

For a long time, the use of antibiotics in the feed helped to consolidate results in terms of performance, health, and economic viability of the activity. Additives that guarantee total replacement of antibiotics have not yet been developed. With the restriction of the use of growth-promoting antibiotics, basic management practices, which for some times were neglected, should be adopted, as well as changes in nutritional strategies.

In this scenario, our objective was to evaluate the replacement of the growth promoting antibiotic in the feed by associated additives and to attenuate the effects of high production densities with the use of new sources of amino acids.

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CHAPTER 2

ARTICLE 1 - BLEND OF ORGANIC ACIDS AND POLYPHENOLS AS A SUBSTITUTE FOR GROWTH PROMOTING ANTIBIOTICS GUARANTEES THE PERFORMANCE AND INTESTINAL HEALTH OF PIGLETS IN THE NURSERY PHASE

ARTIGO FORMATADO DE ACORDO COM AS NORMAS DA REVISTA CIENTÍFICA LIVESTOCK SCIENCE

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RESUMO

O objetivo deste estudo foi avaliar os efeitos de ácidos orgânicos (ácido cítrico, lático e benzóico) associados a polifenóis (do extrato de própolis e do extrato cítrico) na resposta imune celular e humoral, morfologia, capacidade antioxidante na mucosa jejunal, produção de ácidos graxos de cadeira curta (AGCC) no ceco e microbiota colônica em leitões desmamados. Quatrocentos e setenta e seis suínos machos castrados e marrãs foram desmamados (6,21 ± 1,16 kg; cerca de 23 dias de idade) e distribuídos em quatro tratamentos delineamento inteiramente casualizado, com quatro tratamentos: dieta basal sem aditivos antimicrobianos (NC); dieta basal com 120 ppm de halquinol (PC); NC + 0,05% de blend com ácidos orgânicos associados a polifenóis (OAP1); NC + 0,10% da mesma mistura (OAP2). Os porcos foram pesados 0, 10, 14, 28 e 42 dias no experimento. A incidência de diarreia foi avaliada diariamente em todo o período. No dia 10, foram coletadas amostras de sangue, e esses animais foram eutanasiados para coleta de amostras de jejuno, conteúdo cecal e cólon. Na fase inicial II (d 28 a 42), os suínos dos tratamentos PC e OAP2 apresentaram maior peso corporal (PC) (P = 0,021) e ganho médio diário (GMD) (P = 0,002), bem como para todo o período, GMD (P = 0,012). Os porcos NC tiveram a maior incidência de diarreia durante a fase inicial I (P = 0,001), em relação ao período global OAP1 teve a menor incidência de diarreia (P = 0,037). O grupo OAP2 reduziu a contagem de neutrófilos (P = 0,047). Quando comparado com o grupo NC e PC, a suplementação de OAP2 aumentou significativamente (P = 0,003) a glutationa S-transferase (GST) na mucosa jejunal. Nas análises do microbioma, observou-se que os grupos OAP1 e NC apresentaram microbiota colônica semelhante quando comparados aos leitões PC e OAP2. Um aumento significativo no número de unidades taxonômicas operacionais (UTOs) foi observado no grupo NC. Em geral, os gêneros mais abundantes foram Lactobacillus, Megasphaera, Streptococcus, Clostridium sensu stricto 1, Prevotella, Roseburia e Subdoligranulum. Em conclusão, a suplementação com 0,1% do blend com ácidos orgânicos associados a polifenóis pode substituir antibióticos promotores de crescimento e aumentar os parâmetros redox jejunais, modificar o microbioma do cólon e melhorar o desempenho de crescimento de leitões na fase de creche.

Palavras-chave: Aditivos. Saúde intestinal. Microbioma. Leitões.

Blend of organic acids and polyphenols as a substitute for growth promoting antibiotics guarantees the performance and intestinal health of piglets in the nursery phase

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ABSTRACT: The objective of this study was to evaluate the effects of organic acids (citric, lactic, and benzoic acid) associated with polyphenols (propolis and citric extract) on cellular and humoral immune response, morphology, antioxidant capacity in the jejunal mucosa, production of short chain fatty acids (SCFA) in the cecum and colonic microbiota in weaned piglets. Four hundred and seventy-six barrows and gilts were weaned (6.21 ±1.16 kg; 23d of age) and assigned to four treatments in a completely randomized design, with four treatments: basal diet without antimicrobials additives (NC); basal diet with 120 ppm of halquinol (PC); NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); NC + 0.10% of same blend (OAP2). The pigs were weighed 0, 10, 14, 28, and 42d into the experiment. The incidence of diarrhea was assessed daily in the entire period. On day 10, blood samples were collected, and these animals were euthanized to collect jejunal samples, cecal and colon content. In the starter II phase (d 28 to 42), the pigs of the PC, and OAP2 treatments showed a greater body weight (BW) (P = 0.021) and average daily gain (ADG) (P = 0.002) as well as for the entire period, ADG (P = 0.012). The NC pigs had the greatest incidence of diarrhea during the starter I phase (P = 0.001), regarding the overall period OAP1 had the lowest diarrhea incidence (P = 0.037). The OAP2 group reduced the counts of neutrophils (P = 0.047). When compared with the NC and PC group, OAP2 supplementation significantly increased (P = 0.003) the glutathione S-transferase (GST) in the jejunal mucosa. In the microbiome analyses, it was observed that the OAP1 and NC groups had similar colonic microbiota when compared to the PC and OAP2 piglets. A significant increase in the number of operational taxonomic units (OTUs) was observed in the NC group. In general, the most abundant genera were *Lactobacillus*, *Megasphaera*, *Streptococcus*, *Clostridium sensu stricto 1*, *Prevotella*, *Roseburia* and *Subdoligranulum*. In conclusion, supplementation with 0.1% of the blend with organic acids associated with polyphenols can replace growth promoting antibiotics and improve jejunal redox parameters, modify the colon microbiome and improve the growth performance of piglets in the nursery phase.

Keywords: Additive, gut health, microbiome, piglets.

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1. Introduction

Weaning is one of the most challenging practices in swine production (Weary et al., 2008) and consequently, is common to observe a great incidence of diarrhea in the first weeks after weaning (Pluske et al., 2018a). This fact contributes to the substantial use of antibiotics in the swine production system (Albernaz-Gonçalves et al., 2021). Antibiotics as growth promoters and prophylactic use have guaranteed growth rates and aided in the prevention of diseases in swine (Van Boeckel et al., 2015). However, the continued and often indiscriminate use of antibiotics has significantly contributed to the increase in the emergence of multidrug-resistant pathogens, which raises concerns for animal as well as human health (Tang et al., 2017; Landers et al., 2012). Thus, the pressure to withdraw antibiotics from animal production has increased in recent years (Liu et al., 2018).

In order to avoid the negative effects of removing antibiotics from swine diets, numerous changes, especially in handling and adoption of differentiated nutritional strategies, will be increasingly necessary (Kil and Stein, 2010). As a result, the use of alternative additives to antibiotics is gaining more and more space and needs to be validated (Heo et al, 2013; Liu et al. al., 2018). Different additives have different functions and can have synergistic action when used together and this has drawn the attention of researchers to the development of research with blends of additives (Wang et al., 2021a; Lee et al., 2021).

In this scenario the use of organic acids emerges as an alternative to antibiotics. One of the most important mechanisms of action of organic acids is through its dissociation which lowers the pH (Canibe et al., 2001). Through acidification of the environment, organic acids can enhance the activity of enzymes such as trypsin and pepsin (Long et al., 2018), favor the development of beneficial bacteria, improving the digestibility of nutrients (Suiryanrayna and Ramana, 2015). Another mechanism of action of the organic acids is through their capacity to enter the bacterial cells in an undissociated form and cause alterations in the metabolism and osmotic pressure of the cell (Tugnoli et al., 2020). As a result of the benefits to the digestive process, there are several studies that report improved performance of weaned piglets supplemented with different organic acids (Lei et al., 2017; Tsiloyiannis et al., 2001). Anyway, it is interesting to emphasize that the results of the inclusion of organic acids differ according to the dose used, animal age, duration of supplementation, and association with other acids and additives (Lallès et al., 2009).

Propolis and citric extract are rich in polyphenols, mainly flavonoids and phenolic acids (Gonçalves et al., 2018), which have antimicrobial, anti-inflammatory, and antioxidant

properties (Coelho et al., 2010). *In vitro* experiments report that polyphenols are able to reduce the expression of pro-inflammatory cytokines, such as IL-1B, TNF-alfa, and IFN-y (Comalada et al., 2005; Cui et al., 2014; Serra et al., 2013). Some authors also report improvement in antioxidant capacity as the dose of flavonoids used is increased (Myhrstadm et al., 2002; Türkan et al., 2018; Jia et al., 2020). Other studies have reported that different flavonoids such as quercetin (Sánchez de Medina et al., 1996), rutoside (Gálvez et al., 1997), hesperidin (Crespo et al., 1999), and morin (Ocete et al., 1998) improve the absorptive function and integrity of the intestinal barrier leading to fewer symptoms of diarrhea, which are frequent in intestinal inflammation.

There are few studies in the literature reporting the effects of the inclusion of organic acids associated with polyphenols on the growth performance of pigs, as well as their possible action on intestinal health. Our hypothesis is that the use of a mixture with polyphenol-associated organic acids (OAP) can replace growth promoting antibiotics in nursery diets and alter the intestinal health of weaned piglets. Therefore, the aim of this study was to evaluate the effects of OAP on cellular and humoral immune response, morphology, antioxidant capacity in the jejunal mucosa, production of SCFA in the cecum, and colonic microbiota in weaned piglets.

2. Materials and methods

The experiment was conducted at the nursery facility of Animalnutri Research Center located within a commercial pig farm in Patos de Minas, Brazil. The protected blend, of organic acids (citric, lactic, and benzoic acid) associated with polyphenols (from propolis and citric extract) were provided by Quinabra (São Paulo, Brazil). Animal procedures were consistent with the Guide for the Care and Use of Animals in Agricultural Research and Teaching FASS (2010).

2.1 Animals, experimental design, and housing

A total of two hundred eighty-eight barrows and two hundred eighty-eight gilts (DanBred sows and LQ1250 sires) with an average initial body weight of 6.21 ± 1.16 kg (23 ± XX d of age) were randomly allocated in a completely randomized design, with four treatments: basal diet without antimicrobials additives (NC); basal diet with 120 ppm of halquinol (PC); NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); NC + 0.10% of same blend (OAP2). Twelve replicates with 12 pigs per pen. To

simulate a normal farm situation, the composition of the pens was made with 25% light animals, 50% medium animals and 25% heavy animals based on the total animals used in the trial. At day 10 post-weaning, ten pigs per treatment were removed for euthanasia and sample collection.

The pens allowed a floor space of 0.34m² per piglet, had a totally slated plastic floor, and were in an environmentally controlled room. All piglets were provided with feed and water in a semiautomatic feeder and nipple drinkers.

2.2 Diets and experimental procedures

The experimental period of 42 days was divided into four diets. The basal diet was formulated to meet or exceed the nutritional specifications suggested by Rostagno et al. (2017) for pigs during the growing to finishing phases (Supplementary Table S1). The pigs had *ad libitum* access to feed and water throughout the experimental period.

The feed intake and individual body weight were recorded at 0, 10, 14, 28, and 42 days. Based on these data, the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated. For fecal scoring, the feces per pen were assessed daily in the nursery phase and graded as normal feces (no diarrhea) or liquid or pasty stools (presence of diarrhea), following the method of Casey et al. (2007). At the end of each phase, the occurrence of diarrhea was calculated as a percentage of the phase days.

2.3 Sample collections

On day 10 of the trial, blood samples were collected from one piglet per treatment from the ten replicates, totaling 40 animals per collection. The piglets with the closest weight to the pen mean weight were chosen. Blood samples were obtained in tubes without anticoagulant from the anterior vena cava, which were left for 8 hours at 4°C for the removal of blood serum and immediately froze at -20°C for further analyses. For the white blood cells (WBC) counts, blood samples were obtained in EDTA tubes, and the analyses were performed immediately.

The same animals from the blood sample collection were slaughtered. The abdomen was opened, and the gastrointestinal tract was immediately removed. Approximately 2cm segments of the mid-jejunum were quickly isolated, washed with a 0.9% cold saline solution, and fixed in 10% formaldehyde solution for morphology measurements. The jejunal mucosa was scraped with glass microscope slides and snap-frozen in liquid nitrogen and then stored at

-80°C for redox parameter analyses. Additionally, cecal digesta and distal colon contents were aseptically collected and stored at -80°C for SCFA and microbiome analyses, respectively.

2.4 Serum levels of cytokines and white blood cell counts

Serum cytokines tumor necrosis factor alpha (TNF alpha) and interleukin 10 (IL-10) concentrations were determined using the commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits specific for pigs, according to the manufacturer's instructions. For TNF alpha, the kit used was from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and for IL-10 the kit used was from Wuhan Fine Biological Technology Co., Ltd. (Wuhan, Hubei, China). The minimum detectable dose of the TNF alpha and IL-10 kits were 20 pg/ml and 18.75 pg/ml, respectively. The intra- and inter-assay CV of the TNF alpha kit were <10% and <12%, respectively. For the IL-10 kit, the intra- and inter-assay CV were <10% and <12%, respectively. The analyses were performed in triplicate.

The WBC counts (total white blood cells, segmented neutrophils, lymphocytes, and monocytes) were determined (1000 cells/mm^3) using the Sysmex pocH-100iV Diff® hematology analyzer (Sysmex America, Inc., Lincolnshire, IL, USA), using the hydrodynamic focusing impedance-based cell counting methodology.

2.5 Jejunal morphology analyses

The jejunum samples were fixed in 10% formaldehyde solution for 48h and transferred to 70% alcohol solution until the slides were prepared. The histological analyses were performed in paraffin-embedded segments, sectioned at 4μm, and stained with hematoxylin and eosin stain, based on Luna (1968). The slides were photographed using a trinocular microscope (CX31, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil) and digital image capture camera (SC30, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil). The villus height and crypt depth were measured by the AxionVision SE64 4.9.1 software, using 10 well-oriented villi and crypts per tissue. The villus:crypt ratio was calculated.

2.6 Jejunal redox parameters

For the catalase (CAT) and superoxide dismutase (SOD) biochemical analyses, the jejunal samples were homogenized in potassium phosphate buffer solution pH 6.5, at a 1:10 dilution, and centrifuged at a speed of 10,000g for 20 minutes at a temperature of 4ºC. For the GST analyses, the dilution used was 1:30. Catalase activity was quantified according to Aebi (1984). The reaction was carried out using 5 mM hydrogen peroxide in 50 mM phosphate

buffer (pH 7.0) in the presence of cytosolic protein and monitored for 60 seconds at 240 nm in a microplate reader, using the 41 mmolar/cm extinction coefficient. Superoxide dismutase activity was measured as the ability of this enzyme to inhibit pyrogallol auto-oxidation according to the method of Gao et al. (1998). The reaction was performed in a microplate and examined at 440 nm. The amount of enzyme that inhibited the reaction by 50% (IC_{50}) was defined as one unit of SOD, and the enzyme activity was expressed in units of SOD per milligram of total protein (U/mg protein). The GST activity was measured according to the method of Habig et al. (1974), which is based on the ability of this enzyme to conjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with reduced glutathione, forming a thioether that can be measured as an increase in absorbance at 340 nm. Glutathione (GSH) levels were measured according to the method of Sedlak and Lindsay (1968) using tissue that was homogenized with trichloroacetic acid. After centrifugation at 13,750g for 10 min at 4 °C, the absorbance of the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) in methanol was measured at 415 nm in a microplate reader. The individual values were interpolated in a standard curve of GSH and are expressed as μg g tissue^{-1}.

2.7 Cecum short-chair fatty acids

Cecum content was analyzed for SCFA concentration. For this, 2.0 g sample of the contents were added to 4 mL of formic acid (17%) to extract and preserve the SCFA present. Centrifugation was performed at 12,500 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.

2.8 Colon microbiota

Total bacterial DNA was extracted from the samples of cecum contents by using a ZR Fecal DNA MiniPrep® kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions. The extracted DNA was quantified by spectrophotometry at 260nm using a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). To assess the integrity of the extracted DNA, all samples underwent electrophoresis in 1% agarose gel, were stained with a 1% ethidium bromide solution, and visualized with ultraviolet light in a transilluminator.

Thereafter, the variable V4 region of the 16S rRNA gene was amplified using the universal primers 515F and 806R (Caporaso et al., 2011) and KlenTaq Master Mix (Sigma). Amplification controls without a template were employed. The PCR conditions used were: 94°C for 3 min (1 cycle), 94°C for 45 s/50°C for 30 s/68°C for 60 s (18 cycles), and a last step of 72°C for 10 min. The amplicons were quantified with Qubit using an HS dsDNA kit (Invitrogen), diluted to 500 pM, and pooled. Then, 16 pM of pooled DNA were sequenced using MiSeq reagent 500V2. Sequencing was performed using an Illumina MiSeq® sequencer (Illumina) obtaining paired-end reads of 250 bp as described (Caporaso et al., 2011).

For the sequences obtained, the data filtering was completed by removing low quality base, Ns, and joint contaminating sequences, as well as through other processes, and a reliable target sequence was obtained for subsequent analyses. The sequence after splicing was analyzed with the QIIME pipeline (Caporaso et al., 2010; Caporaso et al., 2011), including the extraction of operational taxonomic units (OTUs) and overlapping analyses of OTUs. Operational taxonomic units were clustered with a 97% similarity threshold. To compare the sequences, the 2018 update (SILVA 132) of the SILVA ribosomal sequences database (Yilmaz et al., 2013) was used. To generate the classification of bacterial communities by identifying OTUs, 89,042 reads per sample were used. This was in order to normalize the data and not compare samples with a different number of reads, thus avoiding a taxonomy bias.

2.9 Statistical analyses

The pen was used as an experimental unit for the statistical analysis of growth performance and the pigs were used for the laboratory analyses. The data were analyzed using the SAS software statistical package 9.3 (SAS, Cary, NC), except the microbiome data. The Shapiro-Wilk test was used to evaluate the normality of the parametric data. If the variables did not present a normal distribution, data transformation was performed using PROC RANK. The effects were analyzed using the SAS GLIMMIX procedure appropriate for a randomized block design (initial weight). When the F test ($P < 0.050$) showed a significant difference, Tukey's test was used to compare the means, with a significance level of 0.05. To analyze the incidence of diarrhea, a generalized linear model (binomial analysis) was performed using the GENMOD procedure of SAS 9.3, with a significance level of 0.05. The microbiome analyses were performed using the statistical metagenomics program STAMP: Statistical Analysis of Metagenomic Profiles (Parks et al., 2014). To compare the abundance of the genera identified between treatments, the Kruskal Wallis test ($P < 0.050$) was followed by the Bonferroni

correction test. Only statistically different results were shown. The averages for biodiversity between treatments were compared using the number of OTUs and the Kruskal Wallis test ($P < 0.050$), because they presented a non-parametric distribution according to the Shapiro Wilk test.

3. Results

3.1 Growth performance

The growth performance results of the pigs during the experimental period are presented in Table 1. In the starter II phase (d 28 to 42), the pigs of the PC, and OAP2 treatments showed a greater BW ($P = 0.021$) and ADG ($P = 0.002$) compared to the pigs from the NC, and OAP1 group. When taking in account the entire experimental period, the ADG ($P = 0.012$) of the PC, and OAP2 pigs were greater than NC and OAP1 group.

3.2 Diarrhea incidence

As shown in Figure 1, during the starter I phase (d 14 to 28), the pigs of the NC treatment showed a higher diarrhea incidence compared to the pigs of the other groups ($P = 0.001$). From the overall period (d 0 to 42), the OAP1 treatment showed a lower diarrhea incidence than the NC and PC groups ($P = 0.005$).

3.3 Serum levels of inflammatory cytokines and WBC counts

The serum TNF alpha and IL-10 concentration was not influenced by the treatments ($P > 0.050$, Table 2). The group with OAP2 supplementation had the lowest neutrophils count compared to the pigs of the other treatments ($P = 0.047$, Table 2).

3.4 Jejunal morphology analyses and redox parameters

The villus height, crypt depth, and villus:crypt ratio data are shown in Table 4. There were no differences between the treatments for jejunum morphology ($P > 0.050$). As shown in Table 3, the supplementation with OAP2 group had higher GST enzyme activity than the PC group ($P = 0.003$). The other enzymes were not influenced by the treatments ($P > 0.050$).

3.5 Cecum short-chair fatty acids

Acetic, propionic, and butyric concentrations in cecum contents was not influenced by the treatments ($P > 0.050$, Table 5).

3.6 Colon microbiota

The results of the taxonomic classification, by principal components analysis (PCA), showed that the samples of treatments NC and PC were the most distant among all treatments followed by OAP2 group. Which means they have very different microbiota. Thus, the PC1 axis would explain 34.64% of the changes in the microbiota between the experimental groups (Figure 2). It was observed that samples from the NC and OAP1 groups had similar intestinal microbiota.

Regarding the biodiversity indicators of the bacterial communities (Chao1 index and number of OTus), a significant increase was observed in the pigs of the NC treatment when compared to PC and OAP2 treatments ($P < 0.050$; Figure 3 and 4). For the number of OTUs, greater biodiversity was found for the NC group in relation to the other treatments ($P = 0.006$).

There were identified 210 bacterial genera in the four treatments (Supplementary Sheet 1). In general, the most abundant genera were *Lactobacillus*, *Megasphaera*, *Streptococcus*, *Clostridium sensu stricto 1*, *Prevotella* , *Roseburia*, and *Subdoligranulum*.

Using the Kruskal Wallis test ($P < 0.050$) 85 genera were identified with significantly different abundances between treatments (Supplementary Sheet 2). A detailed comparison of the most abundant genera showed a significant increase in *Megasphaera* and *Prevotella* in OAP1 when compared to the other treatments (Figure 5A and 5B). *Clostridium sensu stricto 1* increased significantly at OAP2 and NC when compared to the other groups (Figure 5C). *Streptococcus* significantly increased in PC when compared to the other groups (Figure 5D). On the other hand, *Roseburia* decreased significantly at PC when compared to the other groups (Figure 5E). *Subdoligranulum* decreased significantly at NC when compared to the other groups (Figure 5F).

4. Discussion

The association of organic acids and other compounds has been widely used as a nutritional strategy to reduce the impact of intestinal disorders caused in post-weaning piglets with consequent loss of performance (Zhai et al., 2020; Lee et al., 2021. A good example of this interaction is benzoic acid and essential oils that together provide benefits for the intestinal health of weaned piglets (Resende et al., 2020; Silva Júnior et al., 2020). Propolis and citric extract is rich in polyphenols, mainly flavonoids and phenolic acids (Salgueiro and Castro, 2016) that are known for their antioxidant, antimicrobial, anti-inflammatory and

immunomodulatory effects, and is largely used as a functional food around the world (Chan et al., 2013). However, there are not many reports in the literature of the association of organic acids and polyphenols, especially in the composition proposed in this study.

The supplementation with OAP2 improved the final BW and ADG during the final and overall period of the nursery phase, as did the addition of the antibiotic growth promoter. Recently, studies using a blend of organic acids showed similar results for growth performance (Wei et al., 2021; Correa et al., 2021) as well as studies with the addition of phenolic compounds in the pig diets (Liu et al., 2022; Cui et al., 2019). However, in these studies there was a difference in FCR justified by the improvement in nutrient digestibility. In our study, it was not possible to observe any difference in the overall FCR, as well as shown by Rajkovic et al. (2021) and Lee et al. (2021). Nonetheless, different experimental conditions associated with low health challenges can cause divergent results in experiments with additives supplementation as an alternative to antibiotics (Wang et al., 2018).

The first two weeks post-weaning is characterized by high rates of diarrhea (Amezcua et al., 2002), an economically important enteric syndrome in pigs due to financial losses caused by high mortality and significant weight loss in surviving piglets (Rhouma et al., 2017). In this study, it was not possible to observe significant differences in the diarrhea incidence during the first two weeks of the nursery phase, possibly for this reason there was no difference in the analysis of intestinal morphology. However, when we analyzed the starter I period (d 14 to 28), the NC group has the highest diarrhea incidence than other groups. Several studies have reported that flavonoids supplementation (Caicedo et al., 2021; Xu et al., 2021; Gonçalves et al., 2018), as well as the addition of organic acids (Resende et al., 2020; Wei et al., 2021; Correa et al., 2021), decreases the number of harmful microorganisms. In addition, there may be a synergistic action between the compounds, with antimicrobial action when they are combined, since phenolic compounds cause significant damage to cell membranes, increasing the efficiency of action of organic acids on bacteria (Omonijo et al., 2018; Pu et al., 2018). Regarding the overall nursery period, the OAP1 group had the lowest diarrhea incidence, but, in our results, the growth performance of piglets fed OAP1 not influenced positively, as well as the NC group. Considering that post-weaning diarrhea is a multifactorial disease (Rhouma et al., 2017), results like ours can be found. However, the microbiome results in our study may help explain this result, in part, where the genera of bacterial communities between NC and OAP1 were very similar.

In this study, the improvement in growth performance at the end of the nursery phase may be related to the modulation promoted in the immune response, redox status, and microbiota at 10 days post-weaning, which possibly contributed to the general health of the animals supplemented with OAP2, having positive effects on the overall of the phase.

The post-weaning period is considered one of the most critical periods in swine production, characterized by a high level of stress (Moeser et al., 2017), high demand on the immune system (Pluske et al., 1997; Pluske et al., 2018b) and high demand on the intestinal redox system (Wei et al., 2017; Zhu et al, 2012), which contributes to intestinal dysfunction. The WBC count are commonly used to estimate the risk of bacterial infection, and an increase in WBC indicates the presence of systemic inflammation (Gordon-Smith, 2009). In the current study, there was a significant difference for neutrophils with the lowest count being for the OAP2 group. Neutrophils, among the cells of the innate immune system, are the most abundant circulating leukocytes (Rosales, 2018) and play an important role in the response to inflammation, as they are the first leukocytes recruited by the local inflammatory response (Kobayashi et al., 2017). In the study by Resende et al. (2020) the use of benzoic acid and essential oils or antibiotics were able to reduce the total neutrophil counts in the post-weaning period. In our study, the CP was not able to reduce the neutrophil count, however in the study in comparison, the use of antibiotics was not as growth promoter. In addition, the anti-inflammatory activity of flavonoids is also related to their ability to reduce neutrophils recruitment (Ferraz et al., 2020; Borghi et al., 2018; Guazelli et al., 2013) and inhibit their activation (Middleton Jr, 1996). Another component related to the immune response is oxidative stress, which was evaluated in this study through antioxidant enzymes. The mechanisms by which organic acids contribute to the improvement of oxidative stress are still unclear. In the study by Diao et al. (2016) 5000 mg/kg benzoic acid supplementation increased SOD and plasma glutathione peroxidase activities in the jejunal mucosa of pigs, which could increase antioxidant capacity. In the study of our research group, the association of benzoic acid and essential oils had no positive effect on antioxidant enzymes (Resende et al., 2020). However, polyphenols, especially flavonoids, are known to improve oxidative stress parameters by direct scavenging of free radicals (Panche et al., 2016) and increasing different enzymatic activities with antioxidant properties (Al-Rejaie et al., 2013). While a direct action of flavonoids in most tissues and compartments, as an antioxidant, is highly unlikely (Fraga, 2007, Galleano et al., 2010), in the gastrointestinal tract the biological relevance of free radical scavenging is realistic (Oteiza et al., 2018). In our study, GST had

the highest enzyme activity for the OAP2 group and the lowest was for the CP group, for the other enzymes was not found any effect. The gut mucosal GST activity gradually increases up to 12 days after weaning, and this is accompanied by a progressive increase in GSH over the same period (Degroote et al., 2012). These results indicate that supplementation with OAP2 was efficient in increasing the enzymatic activity of GST compared to other treatment groups in the first ten days after weaning, on the other hand, some antibiotics may regulate the activity of GST (Türkan et al., 2018). However, there are no studies in the literature related to the use of halquinol.

The interaction between microbiota, intestinal health and pig performance has been widely studied in recent years. However, this relationship is versatile and highly sensitive to several factors, mainly related to diet and host immune system (Duarte and Kim, 2022). Studies with organics acids (Chen et al., 2017; Long et al., 2018), different extract rich in flavonoids (Chen et al., 2020; Xu et al., 2021) and the association between both compounds (Wang et al., 2021a) demonstrated effect on the microbiota of weaned piglets. It is known that high bacterial biodiversity is favorable for general health and productivity (Hildebrand et al., 2013). Unlike this statement, in our study, the groups with the lowest biodiversity at ten days after weaning had better growth performance at the end of the nursery phase. In addition, interestingly, the two treatments with the worst growth performance had very similar microbiota at ten days. The use of antibiotics can reduce the biodiversity of piglets after weaning (Resende et al., 2020; Cui et al., 2019) or increase it (Ma et al., 2022). In the same studies, Ma et al. (2022) and Resende et al. (2020) using organic acids and essential oils found greater biodiversity than the positive control, as well as Cui et al (2019), with flavonoid supplementation.

Our results suggest that the treatments led to significant changes in the composition of the intestinal microbiota of piglets, such changes are evident by the differentiation observed in the composition of the intestinal microbiota. Among the changes that occurred in the constitution of the colon microbiota, there was a significant increase in *Prevotella and Megasphaera* in OAP1 when compared to the other treatments. Previous studies have correlated *Prevotella* with beneficial effects on the host, such as resistance to intestinal inflammatory processes (Dziarski et al., 2016), as well as other studies have correlated increased *Prevotella* with low feed efficiency (Wang et al., 2021b). In the study by Chen et al 2020, the supplementation of an extract rich in alkaloids and flavonoids was able to increase the relative abundance of *Megasphaera*, in contrast Cui et al (2019) found a reduction in the

relative abundance of *Megasphaera* with supplementation of a flavonoid-rich herbal extract. The genus *Megasphaera* is positively correlated with the production of SCFA (Long et al., 2020). Among SCFAs, propionate and butyrate are most often considered to be beneficial to human health (Mukherjee et al., 2020), however, we found no difference in our assessment. Despite all the apparent benefits, the OAP1 group presented one of the worst performances at the end of the test, making it evident that the microbiome is multi-interactive and undergoes changes throughout the animal's life (Ke et al., 2019).

The most expressive changes were observed in the PC group, a significant increase was observed in the genera *Streptococcus*, *Campylobacter*, *Helicobacter*, and *Klebsiella* when compared to the other groups. All these bacterial genera have been related as opportunistic pathogens, as well as etiological agents of zoonoses (Goyette-Desjardins et al., 2014) and, sometimes, as responsible for gastrointestinal infection processes that lead to intestinal dysbiosis (Carrique-Mas et al., 2014). Additionally, *Roseburia* decreased significantly in this group. It is well known that groups of the *Roseburia* genus are an important group of butyrate-producing bacteria (Rivière et al., 2016) which in turn have beneficial effects on intestinal health (Dou et al., 2022), beyond to being correlated with the process of resistance to dysbiosis (Machiels et al., 2013). Dysbiosis is a recognized feature of inflammatory bowel disease, which is generally associated with a decrease in the diversity of intestinal microflora (Gresse et al., 2017), less abundance of the phylum *Firmicutes*, an increase or decrease in certain members of the phylum *Bacteroidetes*, and often an increase in some representatives of the phylum *Proteobacteria* (Huttenhower et al., 2014), a profile that was subtly observed in the PC group together with a lower richness of the microbiota. However, the PC group was one of the best growth performers at the end of the trial compared to the other groups, but at ten days of evaluation there was no difference between the groups. This result may infer that there was not enough challenge to impair the performance of the animals and may also be evidence of microbial resistance to growth-promoting antibiotics. In contrast, *Roseburia* increased significantly in OAP1 and OAP2 when compared to group PC and *Faecalibacterium*, from the phyla *Firmicutes*, had higher abundance for the OAP2 group when compared to other groups. Similar results were found by Wu et al. (2020) with flavonoid supplementation in rats. It is generally known that the genus *Faecalibacterium* is one of the main producers of butyrate in the intestine (Ferreira-Halder et al., 2017). Changes in *Faecalibacterium* abundance have been linked to dysbiosis in several human disorders (Miquel et al., 2013). Within the genus, the most studied species is *Faecalibacterium*

prausnitzii, a Gram-negative, strictly anaerobic bacillus that produces butyrate by fermentation (Duncan et al., 2002). In addition, metabolites secreted by *Faecalibacterium prausnitzii* were able to block the activation of the NF-κB transcription factor, as well as to stimulate the production of the pro-inflammatory cytokine IL-8 by epithelial cells, resulting in an anti-inflammatory response (Sokol et al., 2008). Other anti-inflammatory activities of this genus were observed in mice with induced colitis, through the increase of metabolites such as salicylic acid which in turn decreased the production of IL-8 (Miquel et al., 2015). In contrast, in our evaluation, as well as the results of Wang et al. (2018), there was no difference for the evaluated cytokines.

In our study, it was possible to observe that the blend acted in different compartments in the gastrointestinal tract, showing that the technology used to protect the blend was effective. Likewise, Piva et al (2007) showed that the protection of organic acids favors the permanence of the acid in its undissociated form, which promotes its action throughout the intestine (Tugnoli et al., 2020).

The diversity of results makes it evident that more studies should be carried out to elucidate the multiple interactions between microbiota, immune system, and additives, especially in studies like ours, with new approaches to additive blending. The different types of diet, blend of additives and experimental conditions favor the emergence of divergent results, mainly in relation to the microbiota. Studies with longer collection times throughout the nursery phase help to better interpret the results, especially in tests of associated additives, where the effects can be complementary.

5. Conclusions

In conclusion, supplementation with 0.1% of the blend with organic acids associated with polyphenols, improved intestinal redox parameters, modifies the colon microbiome, and improves the growth performance of piglets in the nursery phase. The results indicate that this combination may be an alternative feed additive to growth promoting antibiotics.

Conflict of interest

The authors of the present study declare that they have no competing interests.

CRediT authorship contribution statement

Rhuan Filipe Chaves: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Maíra Resende: Investigation, Formal

analysis, Writing – review & editing. Gabriel Martins: Writing - review & editing.; Ygor Henrique de Paula: Writing - review & editing; Aline Maria Silva Barbosa: Investigation, Writing - review & editing. Kenio de Gouvêa Cabral: Conceptualization, Visualization, Supervision, Writing - review & editing, Funding acquisition. Vinícius de Souza Cantarelli: Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration.

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Table 1. Effects of experimental diets on growth performance in piglets in the nursery phase.

Item[1]	Treatments[2]				SEM	P-value[3]
	NC	PC	OAP1	OAP2		
Initial BW, kg	6.214	6.213	6.213	6.213	0.060	1.000
Pre-starter I, d 0 to 10						
BW d 10, kg	8.231	8.212	8.204	8.195	0.107	0.996
ADFI, kg	0.281	0.263	0.268	0.285	0.007	0.131
ADG, kg	0.200	0.192	0.191	0.199	0.010	0.892
FCR	1.437	1.436	1.402	1.458	0.085	0.972
Pre-starter II, d 11 to 14						
BW d 14, kg	9.149	9.242	9.126	9.160	0.102	0.861
ADFI, kg	0.369	0.360	0.357	0.360	0.013	0.918
ADG, kg	0.230	0.252	0.219	0.224	0.014	0.367
FCR	1.542	1.520	1.503	1.557	0.111	0.981
Starter I, d 15 to 28						
BW d 28, kg	11.939	12.207	11.847	12.109	0.204	0.596
ADFI, kg	0.480	0.489	0.481	0.501	0.014	0.665
ADG, kg	0.211	0.220	0.194	0.204	0.008	0.190
FCR	2.244	2.253	2.379	2.416	0.092	0.421
Starter II, d 29 to 42						
BW d 42, kg	19.531[b]	20.322[a]	19.330[b]	20.403[a]	0.288	0.021
ADFI, kg	0.817	0.843	0.817	0.837	0.020	0.718
ADG, kg	0.542[b]	0.579[a]	0.534[b]	0.592[a]	0.012	0.002
FCR	1.489	1.428	1.453	1.419	0.029	0.318
Total phase, d 0 to 42						
ADFI, kg	0.534	0.529	0.523	0.549	0.011	0.377
ADG, kg	0.317[b]	0.335[a]	0.312[b]	0.337[a]	0.006	0.012
FCR	1.686	1.600	1.676	1.626	0.032	0.187

[1]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; SEM, standard error of the mean.

[2]NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend.

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are expressed as means (12 replicates/treatment).

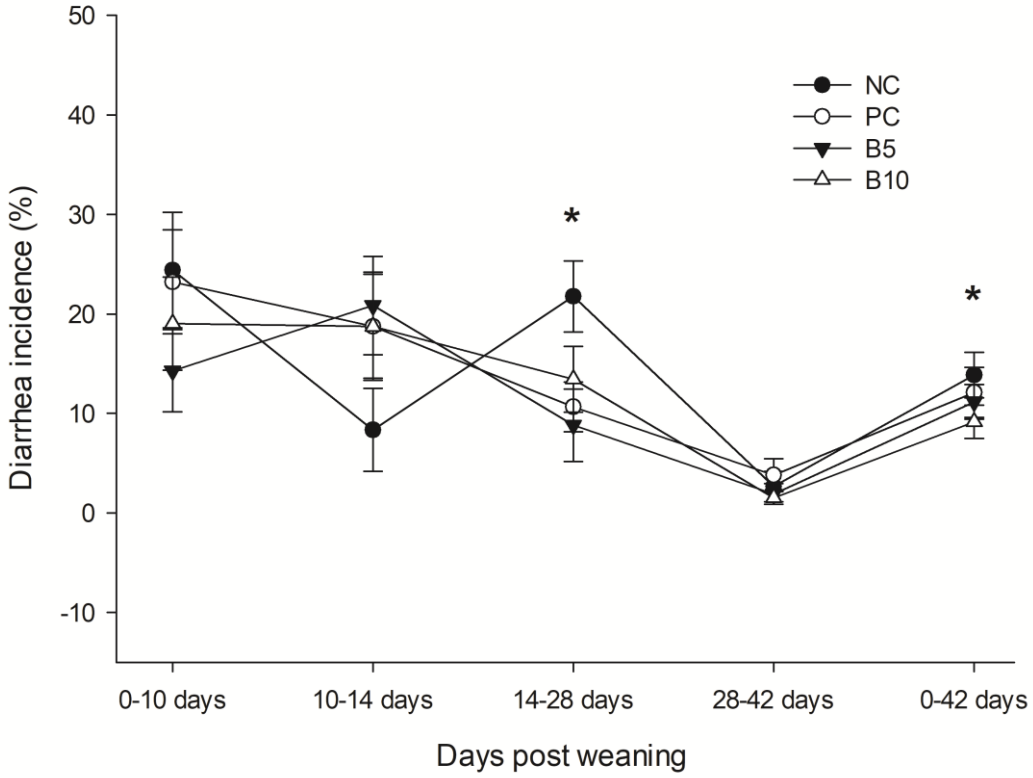


Figure 1. Effect of experimental diets on the diarrhea incidence in piglets in the nursery phase. NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend. Data are expressed as means (12 replicates/treatment) and SEM represented by vertical bars. *Significant differences between groups according to binomial analysis, $P < 0.050$.

Table 2. Effects of experimental diets on serum levels of tumor necrosis factor alpha (TNF alpha) and interleukin (IL)-10 and total white blood cells (WBC) counts of weaned piglets.

Item	Treatments[1]				SEM[2]	P-value[3]
	NC	PC	OAP1	OAP2		
	Serum levels					
TNF alpha, pg/mL	19.00	33.00	21.00	28.00	0.010	0.716
IL-10, pg/mL	3166.20	3323.15	3357.46	2339.53	429.63	0.304
	WBC counts					
WBC total, $x10^3$ cells/mL	17.822	21.217	18.300	16.144	1.111	0.060
Neutrophils, $x10^3$ cells/mL	11.549[a]	12.665[a]	12.210[a]	8.953[b]	1.081	0.047
Lymphocytes, $x10^3$ cells/mL	5.783	7.904	5.758	6.552	0.819	0.209
Monocytes, $x10^3$ cells/mL	0.387	0.424	0.373	0.410	0.074	0.958

[1]NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend.

[2]SEM, standard error of the mean.

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are presented as means (10 piglets/treatment).

Table 3. Effects of experimental diets on intestinal redox parameters of weaned piglets.

Item[1]	Treatments[2]				SEM	P-value[3]
	NC	PC	OAP1	OAP2		
CAT, nmol min^{-1} mg prt^{-1}	21.821	22.644	19.660	19.619	2.067	0.669
SOD, U mg prt^{-1}	141.740	124.010	114.570	142.990	9.011	0.087
GST, mmol min^{-1}mg prt^{-1}	2.188^{bc}	1.795^{c}	2.350^{ab}	2.633^{a}	0.146	0.003
GSH, µg mg $tissue^{-1}$	138.720	134.740	167.090	144.550	16.567	0.533

[1]CAT, catalase; SOD, superoxide dismutase; GST, glutathione-S-transferase; GSH, glutathione; prt, protein; SEM, standard error of the mean.

[2]NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend.

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are presented as means (10 piglets/treatment).

Table 4. Effects of experimental diets on jejunal morphology of weaned piglets.

Item	Treatments[1]				SEM[2]	P-value[3]
	NC	PC	OAP1	OAP2		
Villus height, μm	498.000	510.380	475.600	494.390	20.417	0.687
Crypt depth, μm	315.310	321.510	317.280	318.230	11.788	0.986
Villus:crypta ratio	1.544	1.559	1.552	1.565	0.040	0.984

[1] NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend.

[2]SEM, standard error of the mean.

[3]Data are presented as means (10 piglets/treatment).

Table 5. Effects of experimental diets on cecum short-chair fatty acids of weaned piglets.

Item	Treatments[1]				SEM[2]	P-value[3]
	NC	PC	OAP1	OAP2		
Acetic, mM/mL	58.315	58.713	57.650	60.290	5.103	0.986
Propionic, mM/mL	29.859	24.038	24.949	29.239	2.506	0.247
Butyric, mM/mL	12.430	12.532	12.195	15.184	2.092	0.720

[1] NC, basal diet without additives; PC, basal diet with 120 ppm of halquinol; OAP1, NC + 0.05% of blend with organic acids associated with polyphenols; OAP2, NC + 0.10% of same blend.

[2]SEM, standard error of the mean.

[3]Data are presented as means (10 piglets/treatment).

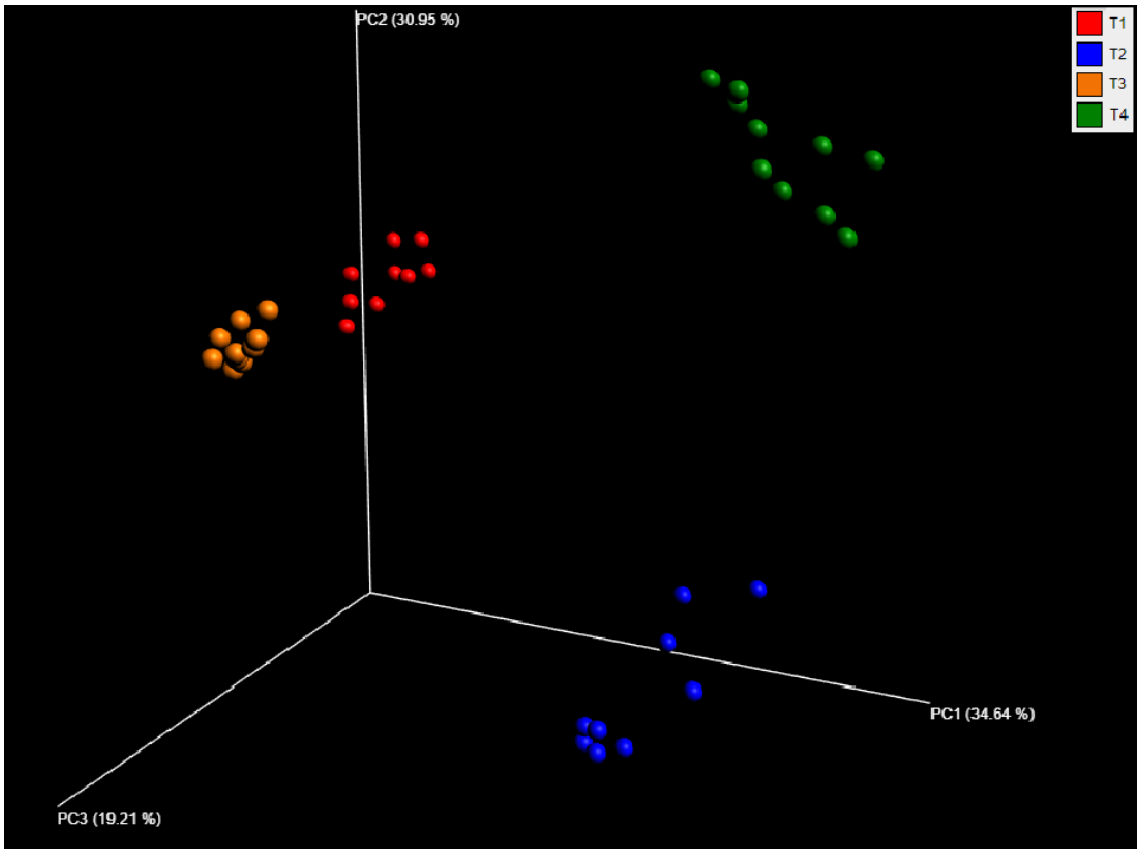


Figure 2. Principal components analysis (PCA) of bacterial community structure between the treatments. Each symbol represents each gut microbiota. T1, basal diet without additives (NC); T2, basal diet with 120 ppm of halquinol (PC); T3, NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); T4, NC + 0.10% of same blend (OAP2). Piglet is an experimental unit; 10 piglets/treatment.

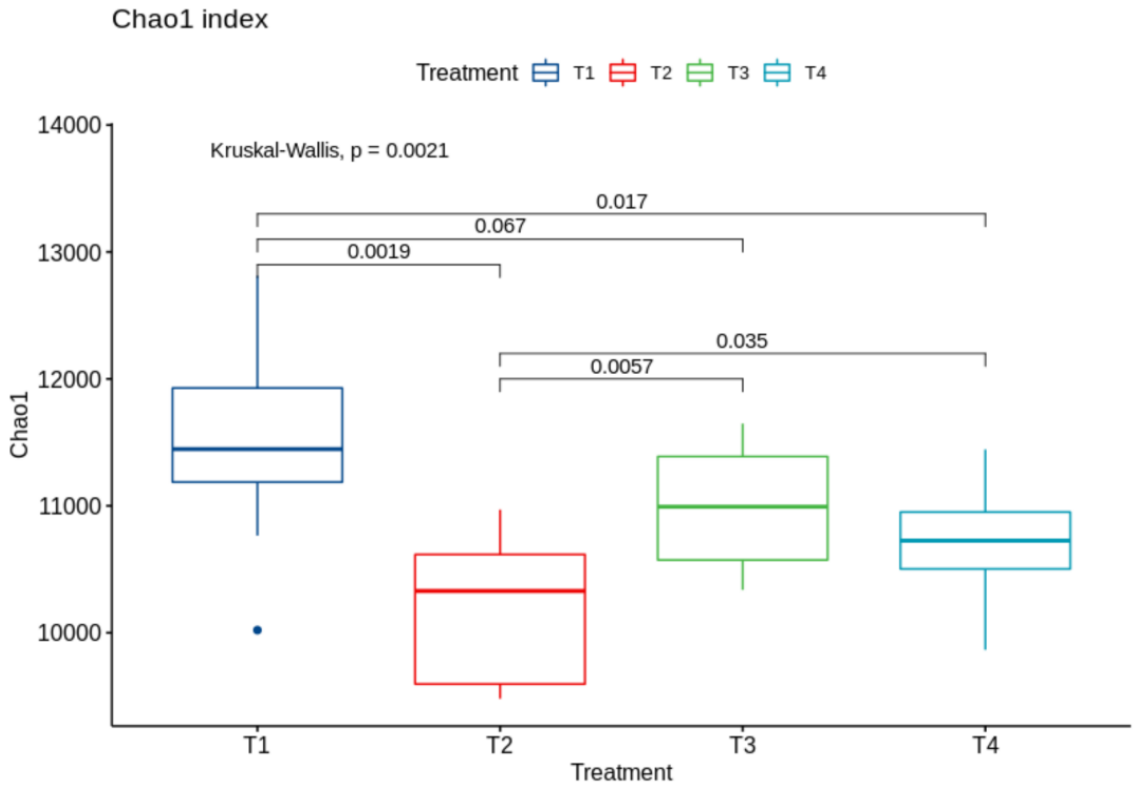


Figure 3. Effects of experimental diets on Chao1 index of weaned piglets. T1, basal diet without additives (NC); T2, basal diet with 120 ppm of halquinol (PC); T3, NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); T4, NC + 0.10% of same blend (OAP2). The indicator means for each experimental group are presented, as well as the significant relationships by the Kruskal Wallis test ($P < 0.050$). Piglet is an experimental unit; 10 piglets/treatment.

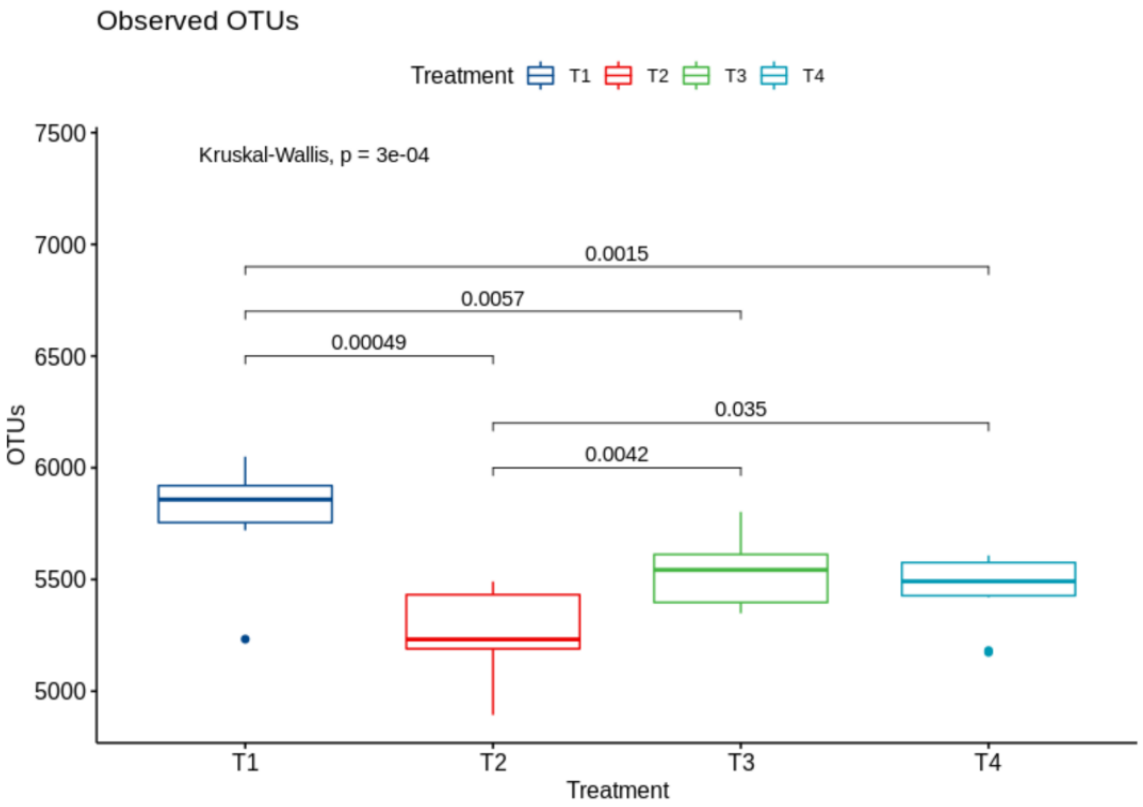
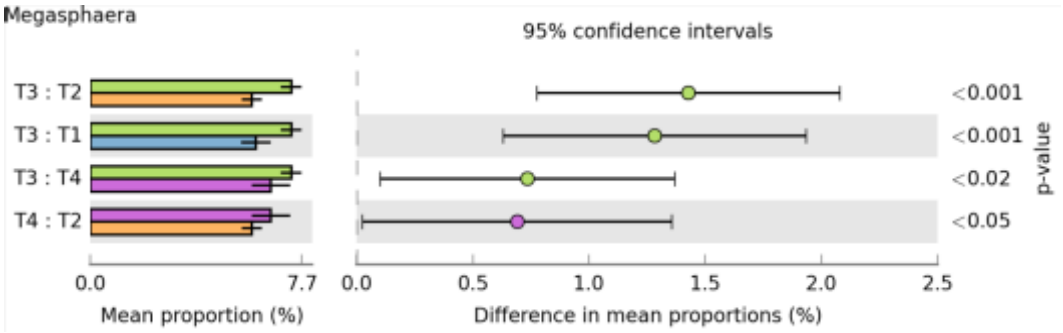
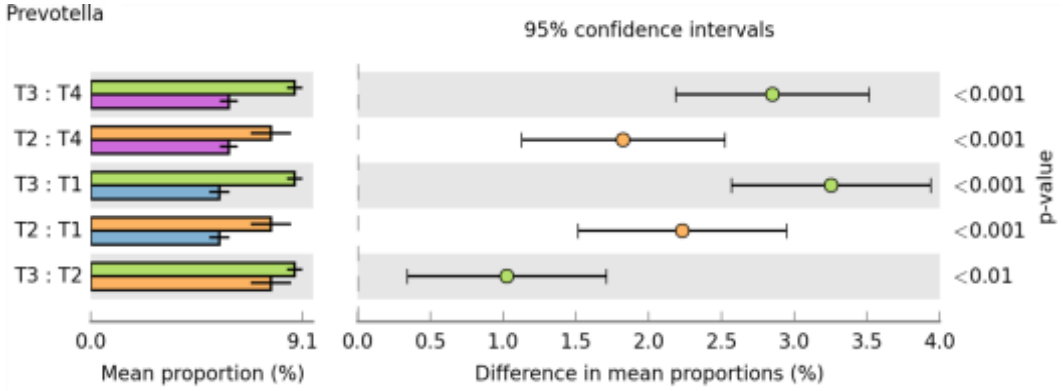


Figure 4. Effects of experimental diets on number of OTUs of weaned piglets. T1, basal diet without additives (NC); T2, basal diet with 120 ppm of halquinol (PC); T3, NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); T4, NC + 0.10% of same blend (OAP2). The indicator means for each experimental group are presented, as well as the significant relationships by the Kruskal Wallis test ($P < 0.050$). Piglet is an experimental unit; 10 piglets/treatment.

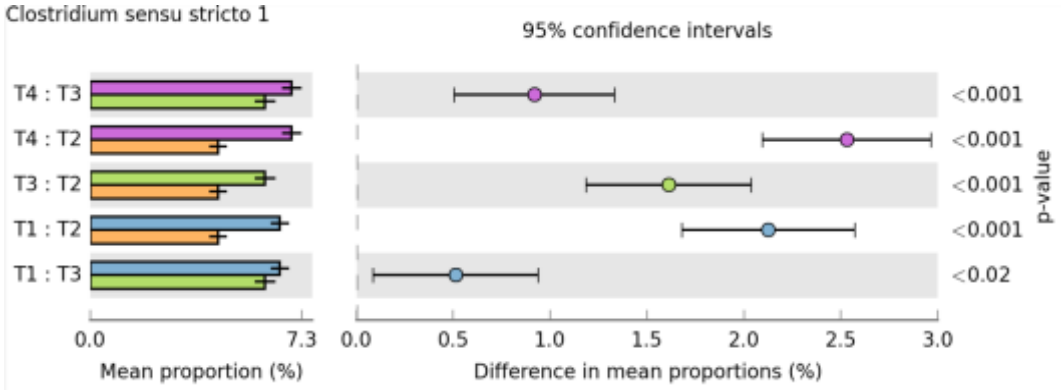
(A)


Megasphaera
95% confidence intervals
T3 : T2
T3 : T1
T3 : T4
T4 : T2
<0.001
<0.001
<0.02
<0.05
p-value
0.0
7.7
Mean proportion (%)
0.0
0.5
1.0
1.5
2.0
2.5
Difference in mean proportions (%)

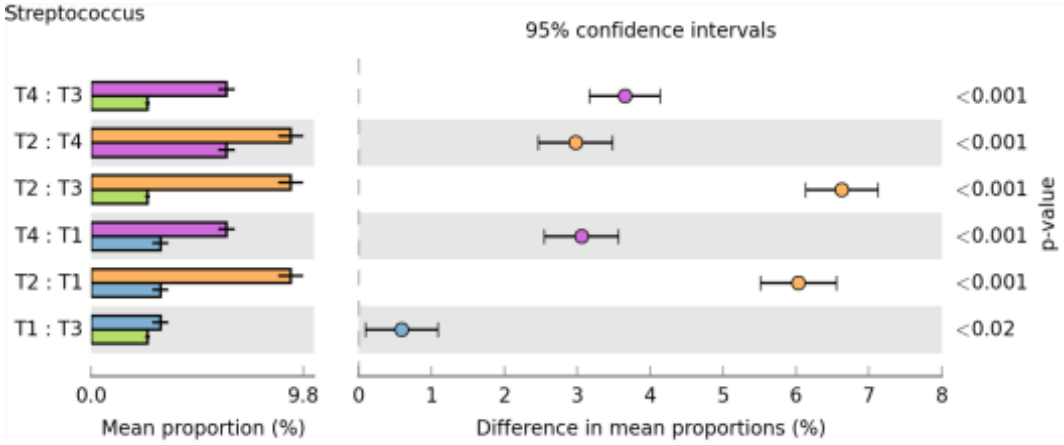
(B)


Prevotella
95% confidence intervals
T3 : T4
T2 : T4
T3 : T1
T2 : T1
T3 : T2
<0.001
<0.001
<0.001
<0.001
<0.01
p-value
0.0
9.1
Mean proportion (%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Difference in mean proportions (%)

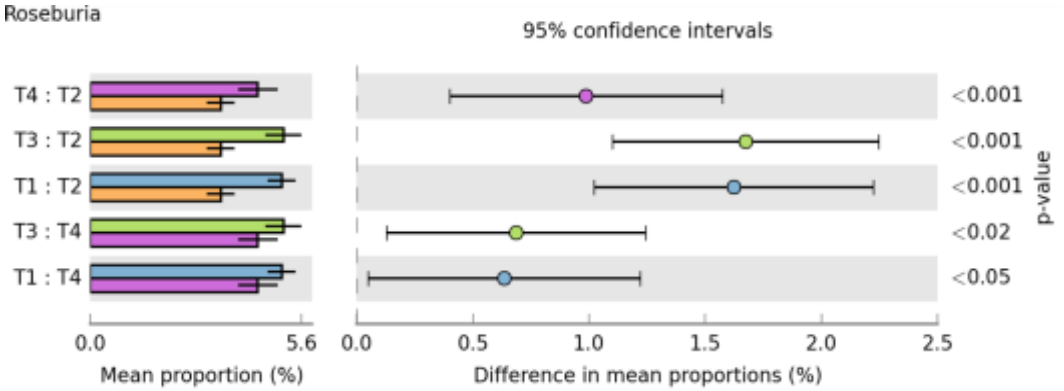
(C)


Clostridium sensu stricto 1
95% confidence intervals
T4 : T3
T4 : T2
T3 : T2
T1 : T2
T1 : T3
<0.001
<0.001
<0.001
<0.001
<0.02
p-value
0.0
7.3
Mean proportion (%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Difference in mean proportions (%)

(D)



(E)



(F)

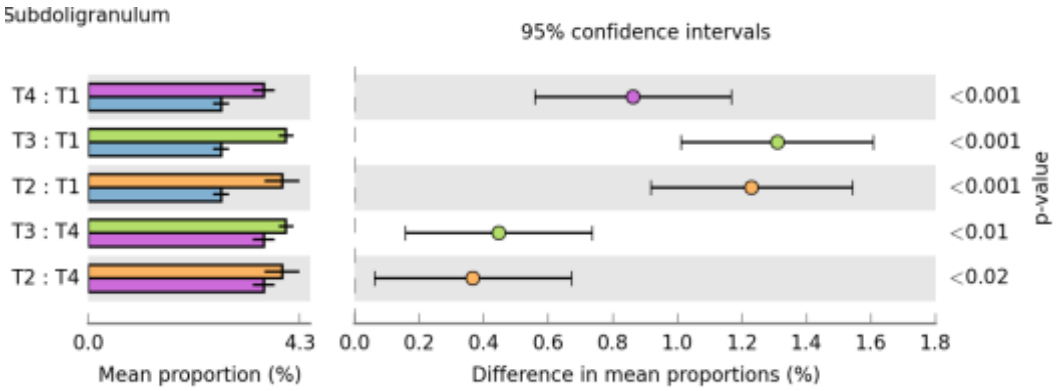


Figure 5. Effect of experimental diets on *Megasphaera* (A), *Prevotella* (B), *Clostridium sensu stricto 1* (C), *Streptoccus* (D), *Roseburia* (E), and *Subdoligranulum* (F) genus relative abundance of weaned piglets, by Kruskal Wallis test with Tukey Kramer's post-hoc test ($P < 0.05$). T1, basal diet without additives (NC); T2, basal diet with 120 ppm of halquinol (PC); T3, NC + 0.05% of blend with organic acids associated with polyphenols (OAP1); T4, NC + 0.10% of same blend (OAP2).Piglet is an experimental unit; 10 piglets/treatment.

Supplementary table S1. Basal diet composition (as-fed basis)

Ingredients. %	Nursery phase			
	Phase I (0-7 days)	**Phase II (7-14 days)**	**Phase III (14-28 days)**	**Phase IV (28-42 days)**
Corn, 7.86% CP[1]	44.036	50.325	60.082	69.239
Micronized integral soybean	7.934	13.632	11.907	2.077
Soybean meal, 46% CP	14.000	16.000	18.000	23.000
Spray-dried plasma	5.000	2.500	-	-
Yeast extract	2.500	-	-	-
Dried whey[*]	16.000	10.000	5.000	-
Dried milk[¥]	8.000	4.000	-	-
Soybean oil	-	-	1.065	2.060
Vitamins and microminerals[£]	0.150	0.150	0.150	0.150
Phytase 10.000 FTU	0.005	0.005	0.005	0.005
Antioxidant	0.020	0.020	0.020	0.020
Flavoring	0.050	0.025	0.025	0.025
Dicalcium phosphate 18.5% P[2]	0.036	0.625	0.805	0.617
Limestone	1.173	0.925	0.839	0.821
Salt	0.253	0.578	0.740	0.658
L-Lysine HCl, 77.5%	0.353	0.521	0.608	0.612
DL-Methionine. 98.5%	0.181	0.225	0.220	0.195
L-Threonine. 94.9%	0.072	0.152	0.180	0.176
L-tryptophan. 99%	0.016	0.028	0.029	0.026
L-valine 96.5%	-	0.044	0.081	0.074
Choline Chloride, 60%	0.013	0.013	0.013	0.013
Inert	0.208	0.208	0.208	0.208
Calculated values				
ME, Kcal/kg	3537	3473	3400	3350
Crude protein, %	21.91	21.25	19.01	17.52
SID[3] Lys, %	1.66	1.63	1.35	1.23
SID Met, %	0.46	0.51	0.48	0.43
SID Met + Cys, %	0.93	0.91	0.74	0.68

SID Thr, %	1.04	1.02	0.79	0.73
SID Trp, %	0.30	0.29	0.22	0.20
Lactose, %	14.44	8.62	3.50	-
Total calcium, %	0.85	0.85	0.80	0.70
Available phosphorus, %	0.45	0.45	0.40	0.33
Sodium, %	0.40	0.40	0.35	0.28

*70% lactose. ¥40%lactose. £Levels per kg of premix. Minerals: 80 g of iron, 10 g of manganese, 200 mg of cobalt, 116 g of zinc, 1228 mg of iodine, 350 mg of selenium, 15 g of cooper. Vitamins: 28,500,000 IU of vitamin A, 5,800,000 IU of vitamin D3, 160,000 IU of vitamin E, 6,000 mg of vitamin K3, 5,400 mg of vitamin B1, 12,000 mg of vitamin B2, 45,000 mg of vitamin pantothenic acid, 7,000 mg of vitamin B6, 70 mg of vitamin B12, 75,000 mg of nicotinic acid, 7,000 mg of folic acid, 850 mg of biotin.

[1]CP = Crude protein; [2]P = phosphorus; [3]SID = standardized ileal digestible.

CHAPTER 3

ARTICLE 2 - EFFECT OF DIFFERENT TRYPTOPHAN SOURCES AT DIFFERENT REARING DENSITIES IN THE NURSERY PHASE ON THE INTESTINAL HEALTH OF PIGLETS

ARTIGO FORMATADO DE ACORDO COM AS NORMAS DA REVISTA CIENTÍFICA LIVESTOCK SCIENCE

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RESUMO

O objetivo foi avaliar a substituição do triptofano cristalino (CTrp) pela biomassa de triptofano (BTrp) e os efeitos no desempenho de crescimento, pontuação fecal, permeabilidade intestinal, parâmetros redox, estado imunológico e cortisol de leitões criados em diferentes densidades de criação. Quatrocentos e oitenta suínos machos castrados e marrãs foram distribuídos aleatoriamente em delineamento de blocos casualizados, com arranjo fatorial 2 × 3 de tratamentos e oito repetições (10 leitões/replica). Duas densidades de criação (0,15 e 0,4 m2/suíno) e três dietas (sem triptofano sintético (Trp), CTrp e BTrp) foram os fatores estudados. O peso corporal foi registrado aos 0, 8, 14, 28 e 42 dias. A pontuação fecal foi classificada como pontuação 1, pontuação 2 e pontuação 3 durante todo o período experimental. No dia 43, amostras de sangue foram coletadas para análises laboratoriais posteriores. Leitões alimentados com CTrp apresentaram maior peso corporal, CRD e GPD do que leitões dos tratamentos deficientes em Trp em todas as fases. Leitões que se alimentaram de BTrp tiveram maior peso corporal do que os tratamentos deficientes de Trp aos 28 e 42 dias e GPD a partir da fase pré-inicial II. Leitões que se alimentaram de BTrp apresentaram maior ADFI apenas na fase inicial I. No período total, os leitões dos tratamentos deficientes em Trp apresentaram CRD e GPD mais baixos, e maior CA do que os leitões alimentados com fontes de Trp. A alta densidade de criação reduziu o PC e GPD e aumentou a CA a partir da fase inicial I. Apenas o fator densidade de criação influenciou no escore fecal dos tratamentos. A partir do pré-inicial II, e considerando o período total, os leitões dos tratamentos com alta densidade de criação tiveram maior porcentagem de pontuação 1 do que os leitões mantidos em densidade normal de criação. A partir do iniciador II, e considerando o período total, observou-se maior porcentagem de escore 2 nos tratamentos com densidade normal de criação. Houve interação dos fatores analisados (densidade de criação e Trp) para a concentração sérica de cortisol, de modo que os leitões do tratamento HD apresentaram maior concentração que os leitões do tratamento ND, assim como do tratamento HD+BTrp. Também houve interação para a concentração de IFN-alfa, sendo que o tratamento HD+BTrp apresentou concentração menor que os demais tratamentos, exceto para o tratamento ND+Ctrp. Em conclusão, a fonte convencional de triptofano pode ser substituída por triptofano de biomassa na fase de creche. Adicionalmente, o uso de biomassa de triptofano pode atenuar as concentrações de indicadores de estresse em animais em altas densidades de produção.

Palavras-chave: Aminoácido. Densidade. Saúde intestinal. Leitões.

Effect of different tryptophan sources at different rearing densities in the nursery phase on the intestinal health of piglets

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ABSTRACT: The objective was to evaluate the replacement of crystalline tryptophan (CTrp) by tryptophan biomass (BTrp) and the effects on growth performance, fecal score, intestinal permeability, redox parameters, immune status, and cortisol of piglets raised at different rearing densities. Four hundred and eighty barrows and gilts were randomly allocated in a randomized block design, with a 2 × 3 factorial arrangement of treatments and eight replicates (10 piglets/replicate). Two rearing density (0.15 and 0.40 m^2/pig) and three diets (without synthetic tryptophan (Trp), CTrp, and BTrp) were the factors studied. The body weight was recorded at 0, 8, 14, 28, and 42 days. Fecal scoring was graded as score 1, score 2, and score 3 during the experimental period. On day 43, blood samples were collected for further laboratory analyses. Piglets fed CTrp had greater BW, ADFI, and ADG than piglets from the Trp-deficient treatments at all phases. Piglets fed BTrp had greater BW than the Trp-deficient treatments at 28 and 42 days and ADG from the pre-starter II phase. Piglets fed BTrp had greater ADFI only in the starter I phase. In the total period, piglets from Trp-deficient treatments had lower ADFI and ADG, and worse FCR than piglets fed Trp sources. The high rearing density reduced the BW and ADG and increased the FCR from the starter I phase. Only the rearing density factor influenced the fecal score of the treatments. From pre-starter II, and considering the total period, piglets from treatments with high rearing density had a higher percentage of score 1 than piglets that were kept at normal rearing density. From starter II, and considering the total period, a higher percentage of score 2 was observed in treatments with normal rearing density. There was an interaction for the analyzed factors (rearing density and Trp) for the serum cortisol concentration, so that the piglets of HD treatment had higher concentration than piglets of ND treatment, as well as HD+BTrp treatment. There was also interaction for the concentration of IFN-alpha, and the HD+BTrp treatment had a lower concentration than the other treatments, except for the ND+Ctrp treatment. In conclusion, the conventional tryptophan source can be replaced by biomass tryptophan in the nursery phase. Additionally, the use of tryptophan biomass can attenuate the concentrations of stress indicators in animals at high rearing densities.

Keywords: Amino acid, density, gut health, pigs.

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1. Introduction

The reduction of crude protein in the diet was largely due to the use of crystalline amino acids, resulting in lower feed costs, and reducing nitrogen excretion (Heo et al., 2009). Among the forms of crystalline amino acids production for its economic and ecological benefits, fermentation is the most prominent process (Humphrey et al., 2020). The first step after fermentation is usually the separation of the desired amino acid from the fermentation biomass (Hermann, 2003). In the fermented biomass, in addition to containing the final amino acid, it also contains amounts of other amino acids, CP, and carbohydrates (Humphrey et al., 2020). Tryptophan is classified as an essential amino acid for pigs and must be provided in the diet (Kaluzna-Czaplinska et al., 2019). Also presenting specific biochemical functions during growth processes, immune regulation, digestion, protein synthesis and anti-stress effect. Thus, the addition of Trp in swine production can improve the performance and health of weaned piglets (Mou et al., 2019).

With the intensification of production in swine, animals are often stocked in high density, which allowed for more efficient management, optimizing the use of facilities and profitability (Rauw et al., 2020). However, due to lack of space in the facilities, the rearing density becomes greater than the minimum recommended. It is known that rearing density affects the environment (Larsen et al., 2017) and pigs exposed to high rearing density suffer severe environmental, social, and psychological stresses (Li et al., 2020). The stress can affect growth performance by increasing the energy demand of animals (Marco-Ramell et al., 2011) and increases competition for feed (Zhang et al., 2013). In addition, chronic stress affects the immune system and health of animals (Moeser et al., 2017).

The research with BTrp for pigs in the nursery phase is limited (Wensley et al., 2019) especially with evaluation of the effects of stress caused by high rearing density. Our hypothesis was that the use of BTrp can replace CTrp and still have additive effects on the health of animals subjected to high rearing densities. Therefore, the aim of this study was to evaluate the replacement of CTrp by BTrp and the effects on growth performance, fecal score, intestinal permeability, redox parameters, immune status, and cortisol of piglets raised at different rearing densities in the nursery phase.

2. Materials and methods

2.1 Animals, experimental design, and housing

The experimental design and procedures were approved by the Ethics Committee on Animal Use of Animalnutri Research Center under Protocol 002/21. The synthetic Trp sources were provided by CJ CheilJedang Corporation (Seoul, Korea).

The experiment was conducted in the nursery facility of the Animalnutri Research Center located within a commercial pig farm in Patos de Minas, Brazil. A total two hundred and forty barrows and two hundred and forty gilts (DanBred sows and LQ1250 sires) with an average initial body weight of 4.89 ±1.00 kg (23 ±XX d of age) were randomly allocated in a randomized block design, with a 2 × 3 factorial arrangement of treatments, totalizing six treatments and eight replicates. Two rearing density (0.15 and 0.40 m^2/pig) and three diets were the factors studied. The experimental unit was of ten piglets. The initial weight was used as a block factor and the pen as an experimental unit. The treatments were: normal rearing density (0.4 m^2/pig) and Trp-deficient, without synthetic Trp (ND); normal rearing density and CTrp (ND+CTrp); normal rearing density and BTrp (ND+BTrp); high rearing density (0.15 m^2/pig) and Trp-deficient, without synthetic Trp (HD); high rearing density and CTrp (HD+CTrp); high rearing density and BTrp (HD+BTrp). For normal rearing density, the free floor area referring to the facilities was used, discounting the area occupied by the feeders used in each pen. For the other treatment (high rearing density), the adequate free floor area was adjusted using divider fences. The feeder space was the same for the treatments. The piglets were allocated in slatted pens equipped with semi-automatic feeder and a single drinking nipple. The feeder space per pen was adequate for the number of animals.

2.2 Diets and experimental procedures

The experimental period of 43 days was divided into four periods. The diets were formulated to meet or exceed the nutritional specifications suggested by the Rostagno et al. (2017) for piglets from the nursery phase, except for Trp in Trp-deficient diets, where synthetic Trp was not added (Supplementary Table S1, S2 and S3). The piglets had *ad libitum* access to feed and water throughout the experimental period. The basal diet did not contain therapeutic antibiotics but did contain copper sulphate and zinc oxide as a growth promoter.

The feed intake and body weight per pen were recorded at 0, 8, 14, 28, and 42 days. Based on these data, the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated. For fecal scoring, the feces per animal were assessed daily and graded as well-formed feces (score 1), sloppy feces (score 2), and diarrhea (score 3), following the method of Marquardt et al. (1999).

2.3 Sample collections

On day 43 of the trial, blood samples were collected from one piglet per replicate, totaling 48 animals. The piglet with the closest individual BW to the group BW was chosen. For detection of fluorescein isothiocyanate dextran (FITC-d, 3-5 kDa) in serum, the piglets received the fluorescent marker Dextran-FITC (Sigma Aldrich Co., St. Louis, MO, USA) orally, after overnight fasting. Within 2.5 hours, blood was collected. Blood samples were obtained in tubes without anticoagulant from the anterior vena cava, followed by centrifugation at 3000x g for 5 min. The serum was separated and immediately froze at -20°C for further analyses (Vicuña et al., 2015).

2.4 FITC-d fluorescence in blood

Fluorescence levels of diluted serum (1:1 in PBS) were measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT), and FITC-d concentration per mL of serum was calculated based on a standard curve.

2.5 Serum inflammatory cytokines

Serum interleukins (IL)-1β; IL-4; IL-6; IL-8; IL-10; IL-12/23p40; interferon (IFN)-alpha; IFN-gamma, and tumor necrosis factor (TNF)-alpha concentrations were determined using the commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits specific for pigs, according to the manufacturer's instructions. The kit used was from Thermo Fisher Scientific Inc. (Cytokine & Chemokine 9-Plex Porcine ProcartaPlex™ Panel 1, Waltham, MA, USA).

2.6 Serum cortisol

Serum cortisol concentration was determined using radioimmunoassay kit (Diagnostic Products, Los Angeles, CA, USA), as previously described (Flynn and Wu,

1997). One mL of the remaining plasma was deproteinized with 1 mL of 1.5 M $HClO_4$ and then neutralized with 0.5 mL of 2.0 M K_2CO_3.

2.7 Serum redox parameters

The lipid peroxidation (LPO) rate was measured by the FOX-2 method (Jiang et al., 1991). This technique determines lipid hydroperoxide synthesis during peroxidation. Absorbance of the supernatant was measured at 560 nm using a microplate reader. The results are expressed as nmol·mg protein^{-1}. Catalase activity was quantified according to Aebi (1984). The reaction was carried out using 5 mM hydrogen peroxide in 50 mM phosphate buffer (pH 7.0) in the presence of cytosolic protein and monitored for 60 seconds at 240 nm in a microplate reader, using the 41 mmolar/cm extinction coefficient. Superoxide dismutase activity was measured as the ability of this enzyme to inhibit pyrogallol auto-oxidation according to the method of Gao et al. (1998). The reaction was performed in a microplate and examined at 440 nm. The amount of enzyme that inhibited the reaction by 50% (IC_{50}) was defined as one unit of SOD, and the enzyme activity was expressed in units of SOD per milligram of total protein (U/mg protein). The GST activity was measured according to the method of Habig et al. (1974), which is based on the ability of this enzyme to conjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with reduced glutathione, forming a thioether that can be measured as an increase in absorbance at 340 nm. Glutathione levels were measured according to the method of Sedlak and Lindsay (1968) using tissue that was homogenized with trichloroacetic acid. After centrifugation at 13,750g for 10 min at 4 °C, the absorbance of the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) in methanol was measured at 415 nm in a microplate reader. The individual values were interpolated in a standard curve of GSH and are expressed as μg g tissue^{-1}.

2.8 Statistical analyses

The pen was used as an experimental unit for the statistical analysis of growth performance and the piglets were used for the laboratory analyses. The data were analyzed using the SAS software statistical package 9.3 (SAS, Cary, NC). The Shapiro-Wilk test was used to evaluate the normality of the parametric data. If the variables did not present a normal distribution, data transformation was performed using PROC RANK. The effects were analyzed using the SAS MIXED procedure appropriate for a randomized block design (initial weight). When the F test ($P < 0.050$) showed a

significant difference, Tukey's test was used to compare the means, with a significance level of 0.050. To analyze the score fecal, a generalized linear model (binomial analysis) was performed using the GENMOD procedure of SAS 9.3, with a significance level of 0.050.

3. Results

3.1 Growth performance

The growth performance results during the experimental period are presented in Table 1. There was an interaction for the analyzed factors (rearing density and Trp sources) for the FCR from 0 to 8 days. The HD+BTrp treatment showed a worse FCR than the HD+CTrp, ND+CTrp, and ND+BTrp treatments ($P = 0.001$). Piglets fed CTrp had higher BW, ADFI, and ADG than piglets from the Trp-deficient treatments at all phases. Piglets fed BTrp had higher BW than the Trp-deficient treatments at 28 and 42 days ($P < 0.005$) and ADG from the pre-starter II phase (d 9 to 14) ($P = 0.001$). Piglets fed BTrp had higher ADFI only in the starter I phase (d 15 to 28) ($P = 0.001$). In the total period, piglets from Trp-deficient treatments had lower ADFI ($P = 0.001$) and ADG ($P < 0.001$), and higher FCR than piglets that fed Trp sources ($P < 0.001$). The high rearing density reduced the BW and ADG, and increased the FCR ($P < 0.050$) from the starter I phase.

3.2 Fecal score

As shown in Table 2, only the rearing density factor had an effect on the fecal score. From pre-starter II phase (d 9 to 14), and considering the total period, piglets from treatments with high rearing density had a higher percentage of score 1 (well-formed feces) than piglets that were kept at normal rearing density. From starter II (d 29 to 42), and considering the total period, a higher percentage of score 2 (sloppy feces) was observed in treatments with normal rearing density.

3.3 Serum FITC-d, inflammatory cytokines, cortisol, and redox parameters

There was no effect of treatments on serum FITC-d levels, as well as for serum redox parameters and serum IL-8 and IL-12 ($P > 0.050$, Table 3). The results for the other cytokines were not presented because they had detectable levels below the minimum value required by the kit. There was an interaction for the analyzed factors

(rearing density and Trp) for the serum cortisol concentration (P = 0.029), so that the piglets of HD treatment had higher concentration than piglets of ND treatment, as well as HD+BTrp treatment (Table 3). There was also interaction for the concentration of IFN-alpha (P < 0.001), and the HD+BTrp treatment had a lower concentration than the other treatments, except for the ND+Ctrp treatment (Table 3).

4. Discussion

The best-known studies with amino acid-containing biomass were conducted with L-Lysine HCL and L-Lysine sulfate with biomass in nursery, growing and finishing pigs, which demonstrate an equality in bioavailability (Smiricky-Tjardes et al., 2004; Liu et al., 2007; Htoo et al., 2016; Li et al., 2019; Palencia et al., 2019). Additionally, recently, some studies have been developed with the aim of evaluating the fermentation biomass of other types of amino acids (Oliveira et al., 2020; Espinosa et al., 2021; Oliveira et al., 2019). This interest is due to the fermentative biomass that contains crude protein, other amino acids, and crude energy (Sulabo et al., 2013; Almeida et al., 2014). This compound has high potential to be used in swine diets as a substitute for crystalline amino acids and may have additive effects on animal health. However, studies with BTrp for swine are limited and an evaluation of this compound on intestinal health parameters under stress conditions has not yet been evaluated.

The current NRC (2012) recommendation ratio between SID Trp and SID lysine (Lys) in the diet of piglets up to 20 kg is 16%. The nutritionally challenged diet in our study contained 13%. In the study by Gonçalves et al. (2015), they concluded that diets with a ratio below 18% SID Trp:Lys resulted in negative growth performance when using commercially available CTrp (98.5% Trp). Tryptophan is a nutritionally essential amino acid in mammals and for swine it is a limiting amino acid (Bravo et al., 2013). Dietary Trp-deficiency has been reported to reduce nutritional levels and impair immune functions, increasing animal's susceptibility to fighting infections and consequently high rates of morbidity and mortality are observed (Harden et al., 2016). Thus, the addition of Trp in pig production improves the growth performance and health of weaned piglets (Mou et al., 2019). This may explain the worse performance of the Trp-deficient group compared to the Trp sources in our study. On the other hand, in the present study, the use of L-Trp with dry fermented biomass showed equivalence to commercial crystalline L-Trp, not negatively impacting the growth performance in the

nursery phase, as well as the results found by Wensley et al. (2019) and Oliveira et al. (2019).

The rearing density is an important parameter in swine production for animal growth performance and welfare (Laskoski et al., 2021). Floor space per animal can be expressed as space per animal (m^2/pig) or by equation ($A = k \times BW^{0.667}$) where A represents the floor space allowance and k represents a constant (Gonyou et al., 2006). Following the tolerances stipulated by the European Council Directive (2008), the value of *k* for pigs weighing up to 20 kg is 0.041 and 0.033 is considered the critical value of *k* (Gonyou et al., 2006). In our study, the HD treatment had a space per animal of 0.15 m^2, according to the equation suggested above, this density would only be harmful for animals above 7 kg of BW. Our results show that the differences in rearing density were only possible 14 days after weaning, where the animals had an average BW of 6.8 kg. Thus, it would be expected that there would be no difference between the rearing densities before the 14 days of evaluation. It is known that space limitations can compromise the growth of pigs (Wolter et al., 2003). In the study by Laskoski et al. (2021), as in our study, the ADG during the first two weeks was not impaired by the smaller space available, but from the second week onwards, the ADG decreased as space was not ideal for the animals. Callahan et al. (2017), in a study comparing 0.27m^2 against 0.19 and 0.15m^2 per animal, reported that the greater useful area increased ADG in medium and large females. These results reinforce the need to respect the ideal rearing density to obtain better growth performance and welfare for piglets in the nursery phase, since as the animals grow, the need for space becomes a limiting factor for maximum growth performance. Interestingly, in our study the ADFI was not affected by the space restriction. The same result occurred in the study by Laskoski et al. (2021). The justification was that as the animals grow, the feed intake time increases, but the frequency of trips to the feeder decreases, however, they conclude that the increase in the availability of the feeder can mitigate the effect of the reduced space. This fact may explain why there was no difference in ADFI, since in the present study the feeder used was sufficient to serve all the animals in the pen.

The fecal score results for the different rearing densities were somewhat inconsistent and there are no reports in the literature to justify the results. However, the evaluation was carried out through the observation of the feces present on the floor of the pen (slatted floor), where the higher density of animals can hinder an accurate

assessment. However, in general, there were no significant differences between treatments for severe diarrhea, represented by score 3.

In addition to replace CTrp by BTrp can probably have additive effects in situations where animals are subjected to stress. In our study, diets with sources of Trp were formulated for the same amount of Trp and met the requirement according to Rostagno et al. (2017). Nevertheless, the Trp biomass source was able to reduce serum cortisol and IFN-alpha levels in piglets subjected to high rearing density. It is already known that dietary supplementation of Trp over than requirements ratios can alleviate the stress induced by the density and mixture of pigs, acting in the reduction of plasma concentrations of cortisol, noradrenaline, and cytokines (Liu et al., 2022; Koopmans et al., 2005, Shen et al., 2012). In the brain, Trp is the precursor of serotonin synthesis that can be metabolized in the pineal body to melatonin (Kopmans et al., 2006). Melatonin has been shown to inhibit the release of adrenocorticotropic hormone (ACTH) which, in turn, regulates adrenal cortisol secretion (Rodwell, 1979). Importantly, only about 5% of endogenous serotonin is found in the brain and about 90% is found in the gut (Fernstrom, 2016). Although this serotonin does not cross the blood-brain barrier, it has been suggested that it affects vagus nerve activity, indirectly manipulating brain function (Alam et al., 2017). An increase in inflammatory cytokines, such as TNF-alpha, IFN—alpha and IFN-gamma, leads to an increase in corticotropin-releasing hormone (CRH), triggering greater ACTH excretion and consequently changes in cortisol levels (Reiche et al., 2005). This cascade of reaction is fed back in situations of chronic stress that favors the increase of intestinal permeability of piglets (Moeser et al. 2007). However, it was not possible to observe difference in intestinal permeability in our study. These results indicate that the use of BTrp may have an additive effect when included in the diet of animals under stress. These effects are possibly related to the various compounds found in fermentation biomass. However, more studies need to be carried out to promote the information bank on the use of BTrp and their relationship with animals' health.

5. Conclusions

In conclusion, the conventional tryptophan source can be replaced by biomass tryptophan without harmful impact on the growth performance in the nursery phase. Additionally, the use of tryptophan biomass can attenuate the concentrations of stress in animals at high rearing densities.

Conflict of interest

The authors of the present study declare that they have no competing interests.

CRediT authorship contribution statement

Rhuan Filipe Chaves: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Maíra Resende: Investigation, Formal analysis, Writing – review & editing. Vinícius de Souza Cantarelli: Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration.

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Table 1. Effect of different tryptophan sources at different rearing densities in growth performance of piglets in nursery phase.

Item[1]	Treatments[2]						High	Normal	Deficient	Crystalline	Trp	SEM	Density[3]	Trp	D x T
	HD	HD+CTrp	HD+BTrp	ND	ND+CTrp	ND+BTrp	density	density	Trp	Trp	biomass				
Initial BW	4.893	4.894	4.894	4.894	4.894	4.894	4.893	4.894	4.893	4.894	4.894	0.379	0.324	0.378	0.378
Pre-starter I, d 0 to 8															
BW d 7, kg	5.548	5.703	5.454	5.485	5.705	5.613	5.568	5.601	5.516^{b}	5.703^{a}	5.533^{ab}	0.415	0.585	0.027	0.311
ADFI, kg	0.228	0.241	0.225	0.213	0.230	0.214	0.231	0.219	0.220	0.236	0.219	0.012	0.121	0.175	0.975
ADG, kg	0.079	0.099	0.070	0.073	0.098	0.098	0.083	0.089	0.075^{b}	0.098^{a}	0.084^{ab}	0.009	0.305	0.027	0.092
FCR	2.633^{ab}	2.391^{b}	3.473^{a}	3.060^{ab}	2.248^{b}	2.210^{b}	2.832	2.506	2.846	2.319	2.841	0.224	0.060	0.022	0.001
Pre-starter II, d 9 to 14															
BW d 14, kg	6.608	6.974	6.753	6.693	7.080	6.885	6.778	6.886	6.650^{b}	7.026^{a}	6.818^{ab}	0.488	0.359	0.037	0.986
ADFI, kg	0.323	0.372	0.354	0.350	0.384	0.355	0.350	0.363	0.336^{b}	0.377^{a}	0.354^{ab}	0.020	0.284	0.034	0.680
ADG, kg	0.176	0.230	0.235	0.201	0.230	0.215	0.214	0.215	0.188^{b}	0.229^{a}	0.224^{a}	0.014	0.836	0.001	0.138
FCR	1.918	1.669	1.551	1.748	1.684	1.629	1.713	1.687	1.832^{a}	1.676^{b}	1.590^{b}	0.056	0.586	0.001	0.092
Starter I, d 15 to 28															
BW d 28, kg	10.625	12.380	11.538	11.363	12.604	12.100	11.514^{b}	12.022^{a}	10.993^{b}	12.491^{a}	11.818^{a}	0.808	0.037	<.0001	0.664
ADFI, kg	0.469	0.540	0.534	0.509	0.561	0.540	0.514	0.536	0.488^{b}	0.550^{a}	0.536^{a}	0.031	0.092	0.001	0.574
ADG, kg	0.286	0.389	0.341	0.334	0.394	0.371	0.338^{b}	0.366^{a}	0.309^{c}	0.391^{a}	0.356^{b}	0.024	0.021	<.0001	0.320

FCR	1.708	1.405	1.568	1.525	1.429	1.455	1.560[a]	1.469[b]	1.616[a]	1.416[b]	1.511[a]	0.047	0.037	<0.001	0.086
Starter II, d 29 to 42															
BW d 42, kg	15.675	18.418	17.379	17.233	19.128	18.540	17.157[b]	18.300[a]	16.453[b]	18.772[a]	17.959[a]	1.060	0.001	<0.001	0.546
ADFI, kg	0.795	0.916	0.864	0.850	0.923	0.885	0.858	0.886	0.822[b]	0.919[a]	0.874[ab]	0.046	0.183	0.002	0.598
ADG, kg	0.361	0.430	0.411	0.420	0.465	0.460	0.400[b]	0.448[a]	0.390[b]	0.447[a]	0.435[a]	0.019	<0.001	<0.001	0.589
FCR	2.196	2.126	2.093	2.031	1.961	1.923	2.138[a]	1.971[b]	2.113[a]	2.043[ab]	2.007[b]	0.039	<0.001	0.010	0.996
Total period, d 0 to 42															
ADFI, kg	0.514	0.585	0.561	0.544	0.593	0.565	0.553	0.567	0.528b	0.588a	0.563ab	0.029	0.252	0.001	0.621
ADG, kg	0.258	0.321	0.298	0.294	0.339	0.325	0.292[b]	0.319[a]	0.275[b]	0.329[a]	0.311[a]	0.017	0.001	<0.001	0.597
FCR	2.010	1.818	1.893	1.853	1.756	1.741	1.907[a]	1.783[b]	1.931[a]	1.787[b]	1.817[b]	0.023	<0.001	<0.001	0.057

[1]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; SEM, standard error of the mean.

[2]ND, normal rearing density (0.4 m^2/pig) and deficient Trp, without synthetic Trp; ND+CTrp, normal rearing density and crystalline Trp; ND+BTrp, normal rearing density and Trp biomass; HD, high rearing density (0.15 m^2/pig) and deficient Trp, without synthetic Trp; HD+CTrp, high rearing density and crystalline Trp; HD+BTrp, high rearing density and Trp biomass.

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are expressed as means (8 replicates/treatment).

Table 2. Effect of different tryptophan sources at different rearing densities in fecal score of piglets in nursery phase, in %.

Item[1]	Treatments[2]						High density	Normal density	SEM	Density[3]	Trp	D x T
	HD	HD+CTrp	HD+BTrp	ND	ND+CTrp	ND+BTrp						
Pre starter I, d 0 to 8												
Score 1	27.880	27.880	25.000	25.000	23.080	27.880	26.920	25.960	0.233	0.643	0.964	0.647
Score 2	37.500	41.350	36.540	43.270	43.270	44.230	38.463	40.387	0.204	0.193	0.899	0.827
Score 3	34.620	30.770	38.460	31.730	33.650	27.880	34.617	33.653	0.219	0.351	0.976	0.312
Pre starter II, d 9 to 14												
Score 1	75.000	81.820	77.270	71.590	75.000	63.640	78.030a	76.893b	0.276	0.039	0.243	0.593
Score 2	20.450	13.640	14.770	19.320	17.050	28.410	16.287	15.910	0.311	0.134	0.360	0.235
Score 3	4.550	4.550	7.950	9.090	7.950	7.950	5.683	7.197	0.512	0.202	0.760	0.636
Starter I, d 15 to 28												
Score 1	96.630	98.080	97.120	92.790	94.230	95.190	97.276a	95.996b	0.505	0.005	0.513	0.739
Score 2	3.370	1.920	2.400	7.210	5.770	4.330	2.563	3.843	0.505	0.005	0.399	0.802
Score 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	-	-	-

Starter II, d 29 to 42												
Score 1	100.000	100.000	100.000	98.560	95.190	98.080	100.000a	99.520b	0.582	<0.001	0.086	1.000
Score 2	0.000	0.000	0.000	1.440	4.810	1.920	0.000b	0.480a	0.582	<0.001	0.086	1.000
Score 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	-	-	-
Total period, d 0 to 42												
Score 1	82.890	84.380	82.890	80.100	79.610	80.100	83.386a	82.456b	0.112	0.007	0.936	0.750
Score 2	10.530	9.700	9.210	13.160	13.490	13.820	9.813b	10.690a	0.14	0.001	0.966	0.723
Score 3	6.580	5.920	7.890	6.740	6.910	6.090	6.797	6.850	0.172	0.824	0.868	0.373

[1]Score 1, well-formed feces; Score 2, sloppy feces; Score 3, diarrhea; SEM, standard error of the mean.

[2]ND, normal rearing density (0.4 m^2/pig) and deficient Trp, without synthetic Trp; ND+CTrp, normal rearing density and crystalline Trp; ND+BTrp, normal rearing density and Trp biomass; HD, high rearing density (0.15 m^2/pig) and deficient Trp, without synthetic Trp; HD+CTrp, high rearing density and crystalline Trp; HD+BTrp, high rearing density and Trp biomass.

[3]Different lowercase letters indicate significant differences between groups according to binomial analysis, $P < 0.050$. Data are expressed as means (8 replicates/treatment).

Table 3. Effect of different tryptophan sources at different rearing densities in serum FITC-d, inflammatory cytokines, cortisol, and redox parameters of piglets in nursery phase.

Item[1]	Treatments[2]						SEM	Density[3]	Trp	D x T
	HD	HD+CTrp	HD+BTrp	ND	ND+CTrp	ND+BTrp				
Serum FITC-d, µg/mL	0.755	0.744	0.570	0.520	0.778	0.795	0.118	0.924	0.471	0.085
Serum GSH, µg.µL	182.360	223.420	196.050	207.830	207.630	188.410	16.288	0.790	0.200	0.313
Serum GST, mmol.min^{-1}.mg prt^{-1}	3.825	7.519	7.241	5.768	6.011	6.740	1.440	0.986	0.267	0.502
Serum CAT, nmol.min^{-1}.mg prt^{-1}	3.945	3.343	3.933	4.882	4.390	5.073	1.358	0.282	0.744	0.878
Serum SOD, U.mg prt^{-1}	446.740	399.720	446.240	496.130	445.460	412.300	26.545	0.310	0.105	0.162
Serum LPO, nmol.mg prt^{-1}	44.160	37.963	43.989	42.795	39.273	40.323	3.029	0.603	0.248	0.700
Serum IL-8, pg/mL	93.366	76.146	113.020	151.030	118.700	87.625	27.045	0.240	0.593	0.556
Serum IL-12, pg/mL	429.840	660.670	525.900	668.090	653.340	740.920	134.060	0.195	0.371	0.272
Serum cortisol, mcg/dL	5.676^{a}	4.312^{ab}	3.465^{b}	3.556^{b}	5.482^{ab}	5.213^{ab}	0.802	0.665	0.760	0.029
Serum IFN-alpha, pg/mL	1.552^{a}	1.102^{ab}	0.304^{c}	1.018^{ab}	0.585^{bc}	2.080^{a}	0.332	0.556	0.210	<0.001

[1]FITC-d, fluorescein isothiocyanate dextran; GST, glutathione-S-transferase; GSH, glutathione; prt, protein; CAT, catalase; SOD, superoxide dismutase; LPO, lipid peroxidation, IL, interleukin; IFN, interferon; SEM, standard error of the mean.

[2]ND, normal rearing density (0.4 m^2/pig) and deficient Trp, without synthetic Trp; ND+CTrp, normal rearing density and crystalline Trp; ND+BTrp, normal rearing density and Trp biomass; HD, high rearing density (0.15 m^2/pig) and deficient Trp, without synthetic Trp; HD+CTrp, high rearing density and crystalline Trp; HD+BTrp, high rearing density and Trp biomass.

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are presented as means (8 piglets/treatment).

Supplementary table S1. Trp-deficient diets composition (as-fed basis)

Ingredients, %	Nursery phase			
	Pre-starter I (0-8 days)	**Pre-starter II (9-14 days)**	**Starter I (15-28 days)**	**Starter II (29-42 days)**
Corn, 7.86% CP[1]	45.043	50.159	57.125	65.500
Soybean meal, 46% CP	12.500	15.500	19.500	23.500
Spray-dried plasma	5.000	4.000	2.500	-
Dried whey[*]	17.500	12.500	7.500	-
Dried milk[¥]	7.500	5.000	-	-
Soybean oil	3.500	3.500	4.000	4.000
Sugar	2.500	2.500	2.500	2.500
Yeast extract	2.500	2.500	2.500	-
Vitamins and microminerals[£]	0.150	0.150	0.150	0.150
Phytase 10.000 FTU	0.005	0.005	0.005	0.005
Sodium butyrate	0.200	0.200	0.200	-
Acids blend	0.600	0.500	0.500	-
Antioxidant	0.025	0.015	0.015	0.015
Dicalcium phosphate 18.5% P[2]	0.450	0.850	0.900	1.200
Limestone	0.400	0.400	0.400	0.700
Salt	0.200	0.250	0.300	0.500
Copper sulfate	0.040	0.040	0.040	0.040
Zinc oxide 80%	0.350	0.350	0.250	0.200
L-Lysine HCl	0.579	0.615	0.654	0.719
DL-Methionine	0.255	0.258	0.253	0.266
L-Threonine	0.315	0.320	0.322	0.305
L-valine	0.192	0.195	0.200	0.212
Inert	0.141	0.138	0.132	0.123
Biocholine	0.025	0.025	0.025	0.025
Flavoring	0.030	0.030	0.030	0.030
Calculated values				

ME, Kcal/kg	3598.00	3533.00	3458.00	3460.00
Crude protein, %	18.95	18.85	18.36	17.05
SID[3] Lys, %	1.45	1.43	1.36	1.28
SID Met, %	0.48	0.49	0.47	0.49
SID Met + Cys, %	0.81	0.80	0.76	0.73
SID Thr, %	0.97	0.96	0.91	0.83
SID Trp, %	0.19	0.19	0.18	0.17
SID Val, %	1.00	0.99	0.94	0.88
Lactose, %	15.40	10.85	5.25	-
Total calcium, %	0.74	0.77	0.70	0.75
Available phosphorus, %	0.53	0.55	0.49	0.43

[*]70% lactose. [¥]40%lactose. [£]Levels per kg of premix. Minerals: 80 g of iron, 10 g of manganese, 200 mg of cobalt, 116 g of zinc, 1228 mg of iodine, 350 mg of selenium, 15 g of cooper. Vitamins: 28,500,000 IU of vitamin A, 5,800,000 IU of vitamin D3, 160,000 IU of vitamin E, 6,000 mg of vitamin K3, 5,400 mg of vitamin B1, 12,000 mg of vitamin B2, 45,000 mg of vitamin pantothenic acid, 7,000 mg of vitamin B6, 70 mg of vitamin B12, 75,000 mg of nicotinic acid, 7,000 mg of folic acid, 850 mg of biotin.

[1]CP = Crude protein; [2]P = phosphorus; [3]SID = standardized ileal digestible.

Supplementary table S2. Crystalline Trp diets composition (as-fed basis)

Ingredients, %	Nursery phase			
	Pre-starter I (0-8 days)	**Pre-starter II (9-14 days)**	**Starter I (15-28 days)**	**Starter II (29-42 days)**
Corn, 7.86% CP[1]	45.098	50.213	57.177	65.548
Soybean meal, 46% CP	12.500	15.500	19.500	23.500
Spray-dried plasma	5.000	4.000	2.500	-
Dried whey[*]	17.500	12.500	7.500	-
Dried milk[¥]	7.500	5.000	-	-
Soybean oil	3.500	3.500	4.000	4.000
Sugar	2.500	2.500	2.500	2.500
Yeast extract	2.500	2.500	2.500	-
Vitamins and microminerals[£]	0.150	0.150	0.150	0.150
Phytase 10.000 FTU	0.005	0.005	0.005	0.005
Sodium butyrate	0.200	0.200	0.200	-
Acids blend	0.600	0.500	0.500	-
Antioxidant	0.025	0.015	0.015	0.015
Dicalcium phosphate 18.5% P[2]	0.450	0.850	0.900	1.200
Limestone	0.400	0.400	0.400	0.700
Salt	0.200	0.250	0.300	0.500
Copper sulfate	0.040	0.040	0.040	0.040
Zinc oxide 80%	0.350	0.350	0.250	0.200
L-Lysine HCl	0.579	0.615	0.654	0.719
DL-Methionine	0.255	0.258	0.253	0.266
L-Threonine	0.315	0.320	0.322	0.305
L-valine	0.192	0.195	0.200	0.212
Crystalline L-tryptophan	0.086	0.084	0.080	0.075
Biocholine	0.025	0.025	0.025	0.025
Flavoring	0.030	0.030	0.030	0.030
Calculated values				

ME, Kcal/kg	3598.00	3533.00	3458.00	3460.00
Crude protein, %	18.95	18.85	18.36	17.05
SID[3] Lys, %	1.45	1.43	1.36	1.28
SID Met, %	0.48	0.49	0.47	0.49
SID Met + Cys, %	0.81	0.80	0.76	0.73
SID Thr, %	0.97	0.96	0.91	0.83
SID Trp, %	0.28	0.27	0.26	0.24
SID Val, %	1.00	0.99	0.94	0.88
Lactose, %	15.40	10.85	5.25	-
Total calcium, %	0.74	0.77	0.70	0.75
Available phosphorus, %	0.53	0.55	0.49	0.43

[*]70% lactose. [¥]40%lactose. [£]Levels per kg of premix. Minerals: 80 g of iron, 10 g of manganese, 200 mg of cobalt, 116 g of zinc, 1228 mg of iodine, 350 mg of selenium, 15 g of cooper. Vitamins: 28,500,000 IU of vitamin A, 5,800,000 IU of vitamin D3, 160,000 IU of vitamin E, 6,000 mg of vitamin K3, 5,400 mg of vitamin B1, 12,000 mg of vitamin B2, 45,000 mg of vitamin pantothenic acid, 7,000 mg of vitamin B6, 70 mg of vitamin B12, 75,000 mg of nicotinic acid, 7,000 mg of folic acid, 850 mg of biotin.

[1]CP = Crude protein; [2]P = phosphorus; [3]SID = standardized ileal digestible.

Supplementary table S3. Trp biomass diets composition (as-fed basis)

Ingredients, %	Nursery phase			
	Pre-starter I (0-8 days)	**Pre-starter II (9-14 days)**	**Starter I (15-28 days)**	**Starter II (29-42 days)**
Corn, 7.86% CP[1]	45.043	50.159	57.125	65.500
Soybean meal, 46% CP	12.500	15.500	19.500	23.500
Spray-dried plasma	5.000	4.000	2.500	-
Dried whey[*]	17.500	12.500	7.500	-
Dried milk[¥]	7.500	5.000	-	-
Soybean oil	3.500	3.500	4.000	4.000
Sugar	2.500	2.500	2.500	2.500
Yeast extract	2.500	2.500	2.500	-
Vitamins and microminerals[£]	0.150	0.150	0.150	0.150
Phytase 10.000 FTU	0.005	0.005	0.005	0.005
Sodium butyrate	0.200	0.200	0.200	-
Acids blend	0.600	0.500	0.500	-
Antioxidant	0.025	0.015	0.015	0.015
Dicalcium phosphate 18.5% P[2]	0.450	0.850	0.900	1.200
Limestone	0.400	0.400	0.400	0.700
Salt	0.200	0.250	0.300	0.500
Copper sulfate	0.040	0.040	0.040	0.040
Zinc oxide 80%	0.350	0.350	0.250	0.200
L-Lysine HCl	0.579	0.615	0.654	0.719
DL-Methionine	0.255	0.258	0.253	0.266
L-Threonine	0.315	0.320	0.322	0.305
L-valine	0.192	0.195	0.200	0.212
L-Trp biomass	0.141	0.138	0.132	0.123
Biocholine	0.025	0.025	0.025	0.025
Flavoring	0.030	0.030	0.030	0.030
Calculated values				

ME, Kcal/kg	3598.00	3533.00	3458.00	3460.00
Crude protein, %	18.95	18.85	18.36	17.05
SID[3] Lys, %	1.45	1.43	1.36	1.28
SID Met, %	0.48	0.49	0.47	0.49
SID Met + Cys, %	0.81	0.80	0.76	0.73
SID Thr, %	0.97	0.96	0.91	0.83
SID Trp, %	0.28	0.27	0.26	0.24
SID Val, %	1.00	0.99	0.94	0.88
Lactose, %	15.40	10.85	5.25	-
Total calcium, %	0.74	0.77	0.70	0.75
Available phosphorus, %	0.53	0.55	0.49	0.43

[*]70% lactose. [¥]40%lactose. [£]Levels per kg of premix. Minerals: 80 g of iron, 10 g of manganese, 200 mg of cobalt, 116 g of zinc, 1228 mg of iodine, 350 mg of selenium, 15 g of cooper. Vitamins: 28,500,000 IU of vitamin A, 5,800,000 IU of vitamin D3, 160,000 IU of vitamin E, 6,000 mg of vitamin K3, 5,400 mg of vitamin B1, 12,000 mg of vitamin B2, 45,000 mg of vitamin pantothenic acid, 7,000 mg of vitamin B6, 70 mg of vitamin B12, 75,000 mg of nicotinic acid, 7,000 mg of folic acid, 850 mg of biotin.

[1]CP = Crude protein; [2]P = phosphorus; [3]SID = standardized ileal digestible.