**MAÍRA RESENDE**

# MANAGEMENT OF IN-FEED ANTIBIOTICS FOR SWINE: EVALUATION OF ALTERNATIVE DIETARY STRATEGIES

**LAVRAS - MG**
**2021**

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

Orientador
Prof. Dr. Vinícius de Souza Cantarelli

Coorientadores
Prof. Dr. Rony Antonio Ferreira
Prof. Dr. Márvio Lobão Teixeira de Abreu

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APROVADA em 19 de maio de 2021
Dr. Vinícius de Souza Cantarelli – Universidade Federal de Lavras - UFLA
Dr. Márvio Lobão Teixeira de Abreu – Universidade Federal de Lavras - UFLA
Dr. Ana Paula Peconick – Universidade Federal de Lavras - UFLA
Dr. Cesar Augusto Pospissil Garbossa – Universidade de São Paulo - USP
Dr. Matheus de Oliveira Costa – University of Saskatchewan, Canada

Prof. Dr. Vinícius de Souza Cantarelli
Orientador

**LAVRAS - MG**
**2021**

*Aos meus pais, meus pilares!*

*Rhuan, meu melhor cúmplice!*

*Liz, minha inspiração!*

*Dedico*

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

*Albert Einstein*

## RESUMO

Dois experimentos foram conduzidos para avaliar os efeitos da substituição (parcial ou total) dos antibióticos por aditivos nutricionais em suínos da creche a terminação. O objetivo do estudo 1 foi avaliar a associação do ácido benzoico (AB) e dos óleos essenciais (OE) na resposta imune; morfologia, expressão gênica e capacidade antioxidante na mucosa jejunal; microbiota do conteúdo cecal, no pós-desmame e desempenho dos suínos da creche à terminação. Cento e vinte suínos machos castrados foram desmamados e distribuídos em um delineamento de blocos casualizados: dieta basal (DB) sem aditivos (CN), DB com antibióticos (CP) e DB com 0,3% de BA e OE (AB+OE). Na fase de creche, os leitões do grupo CP apresentaram maior peso e ganho médio diário (GPD) e melhor conversão alimentar (CA) em comparação com o grupo CN, mas semelhantes aos do grupo AB+OE. Na fase de crescimento, o tratamento CP resultou em maior peso e consumo médio de ração diário (CMRD), e na fase de terminação o peso do grupo AB+OE foi semelhante ao do grupo CP e maior que o do grupo CN. No período total, o peso e o GPD dos grupos CP e AB+OE foram semelhantes e superiores aos do tratamento CN. Os leitões AB+OE tiveram a menor incidência de diarreia durante o período de creche. O uso de AB+OE reduziu a contagem de leucócitos totais e neutrófilos. Nas análises do microbioma, observou-se que os grupos AB+OE e CP apresentaram microbiota cecal semelhantes. Um aumento significativo no número de unidades taxonômicas operacionais (OTUs) foi observado no grupo AB+OE. Em conclusão, o uso de AB+EO reduziu a resposta inflamatória e modificou o microbioma cecal no período pós-desmame, resultando em melhora no desempenho de suínos em terminação. O objetivo do estudo 2 foi avaliar o uso de produtos à base de levedura (LEV) e OE em substituição total ou parcial aos protocolos de antibióticos via ração (promotor de crescimento e profilático), tanto em doses recomendadas quanto em superdose dos antibióticos profiláticos, no desempenho e incidência de diarreia em suínos em crescimento e terminação; e na microbiota fecal de suínos em idade de abate. Quatrocentos suínos (20,36±2,64 kg) foram distribuídos em um delineamento em blocos casualizados: dietas com antibióticos profiláticos e promotor de crescimento (ANT); ANT com 30% a mais de antibióticos profiláticos (ANT+30); dietas com menos profiláticos e LEV (ANT+LEV); dietas com menos profiláticos, LEV e OE (ANT+LEV+OE); dietas sem antibióticos com LEV e OE (LEV+OE). Dos 0 aos 14 dias, os suínos dos grupos ANT+30, ANT+LEV e ANT+LEV+OE apresentaram um maior peso e GPD do que os suínos do grupo LEV+OE. Aos 35 dias, os suínos dos tratamentos ANT e ANT+LEV+OE apresentaram maior peso do que o grupo LEV+OE. Aos 105 dias, suínos do grupo ANT tiveram um peso maior que os suínos do grupo LEV+ANT. Durante o período total, os suínos do grupo ANT apresentaram maior GPD e melhor CA do que o grupo LEV+OE. Dos 49 aos 70 dias, os tratamentos ANT+LEV e ANT+LEV+OE mostraram uma incidência de diarreia menor do que o grupo LEV+OE, o que permaneceu durante todo o período. Aos 105 dias, a diversidade alfa da microbiota fecal foi menor nos grupos ANT, ANT+30 e LEV+OE do que a observada para o grupo ANT+LEV+OE. Em conclusão, o uso de LEV e OE (promotor de crescimento e profilático), em substituição parcial aos protocolos de antibióticos via ração, não reduz o desempenho de crescimento, pode substituir os promotores de crescimento de antibióticos e reduzir a uso na ração de antibióticos profiláticos em suínos em crescimento e terminação. O uso de LEV e OE, juntamente com antibióticos profiláticos, aumenta a diversidade microbiana, apesar de apresentar gêneros importantes para ganho de peso em menor abundância. A superdose de antibióticos terapêuticos não melhora o desempenho do crescimento e reduz a diversidade microbiana, o que não o caracteriza como um protocolo preventivo eficiente.



**Palavras-chave:** Aditivos. Suínos. Diarreia. Saúde intestinal. Microbioma.

## ABSTRACT

Two experiments were conducted to evaluate the effects of the replacement (partial or complete) of antibiotics (ANT) by nutritional additives for pigs in the nursery to finishing phase. The objective of study 1 was to evaluate the effects of benzoic acid (BA) and essential oils (EO) on cellular and humoral immune response, morphology, gene expression, antioxidant capacity in the jejunal mucosa, and cecal content microbiota in weaned piglets and growth performance from the nursery to finishing phase. One hundred and twenty barrows were weaned and assigned in a randomized block design: basal diet (BD) without additives (NC), BD with ANT (PC), and BD with 0.3% BA and EO (BA+EO). In the nursery phase, the PC piglets showed a greater body weight (BW) and average daily gain (ADG), and a lower feed conversion ratio (FCR) compared to the NC group, but similar levels to the BA+EO group. In the growing phase, the PC treatment resulted in a greater BW and average daily feed intake (ADFI), and in the finishing phase the BW of the BA+EO group was similar to that of the PC group and greater than that of the NC group. In the total period, the BW and ADG of the PC and BA+EO pigs were similar and higher than those for the NC treatment. The BA+EO pigs had the lowest incidence of diarrhea during the nursery period. The use of BA+EO reduced the counts of total white blood cells (WBC) and neutrophils. In the microbiome analyses, it was observed that the BA+EO and PC groups had similar cecal microbiota when compared to the NC piglets. A significant increase in the number of operational taxonomic units (OTUs) was observed in the BA+EO group. In conclusion, supplementation with BA+EO reduced the inflammatory response and modified the cecal microbiome in the post-weaning period, resulting in an improvement in the growth performance of finishing pigs. The objective of study 2 was to evaluate the use of yeast products (YP) and EO in total or partial replacement to the in-feed antibiotic protocols (growth promoter and prophylactic), both in recommended doses and in overdose of prophylactic antibiotics, on growth performance, diarrhea incidence in the growing-finishing pigs, and fecal microbiota in market hogs. Four hundred pigs (20.36 ± 2.64 kg) were assigned in a randomized block design: diets with prophylactic and growth promoter antibiotics (ANT); ANT with 30% more prophylactic antibiotics (ANT+30); diets with less prophylactic antibiotics and YP (ANT+Y); diets with less prophylactic antibiotics, YP and EO (ANT+Y+EO); and diets free antibiotics and with YP and EO (Y+EO). From 0 to 14d, pigs of the ANT+30, ANT+Y, and ANT+Y+EO treatments showed a greater BW and ADG compared to pigs from the Y+EO group. From 14 to 35d, pigs of ANT+30 and ANT+Y+EO treatments were to be heavier than Y+EO group. At 105d, ANT pigs had a higher BW than the Y+EO group. For the entire period, ADG of ANT pigs were greater and FCR better than Y+EO pigs. From 49 to 70d, ANT+Y and ANT+Y+EO treatments showed a lower diarrhea incidence than Y+EO group, which remained the case during the overall period. At 105d, the alpha diversity of fecal microbiota were lower in ANT+30 and Y+EO groups than observed for ANT+Y+EO group. In conclusion, the use of YP and EO (growth promoter and prophylactic), in partial replacement to the in-feed antibiotic protocols, does not reduce the growth performance, can replace antibiotic growth promotors, and reduce the in-feed use of prophylactic antibiotics in growing-finishing pigs. The use of YP and EO, together with prophylactic antibiotics, increase the microbial diversity, despite having important genera for weight gain in less abundance. Overdose of prophylactic antibiotics does not improve growth performance and reduces microbial diversity, which does not characterize it as an efficient preventive protocol.



**Keywords**: Additives. Pigs. Diarrhea. Intestinal health. Microbiome.

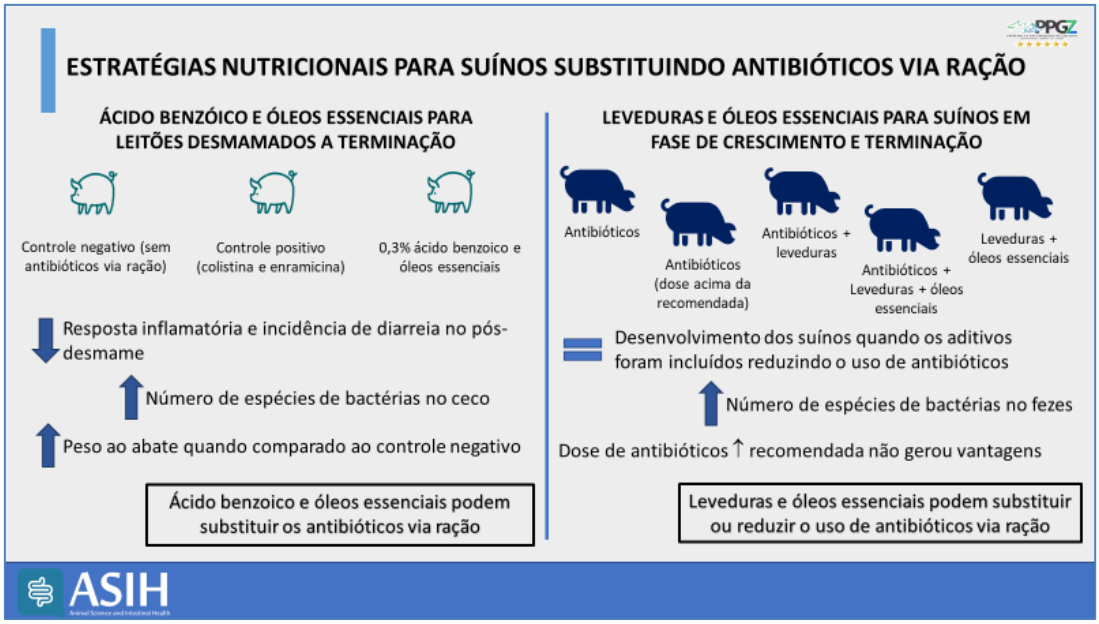
# Estratégias nutricionais para suínos substituindo antibióticos via ração

Elaborado por **Maíra Resende** e orientado por **Vinícius de Souza Cantarelli**

Estratégias nutricionais para minimizar o uso de antibióticos via ração na produção suína têm sido alvo de diversos estudos. Neste caso, são usados aditivos nutricionais que podem substituir estes antibióticos, pois possuem propriedades antimicrobianas ou anti-inflamatórias parecidas. Neste trabalho, diferentes aditivos via ração foram testados em dois experimentos.

No experimento 1, uma associação de ácido benzoico e óleos essenciais foi avaliada como alternativa ao uso de antibióticos na ração. Foi observado que o uso dos aditivos reduziu a resposta inflamatória e aumentou o número de espécies de bactérias presentes no ceco dos leitões recém-desmamados. Estes leitões apresentaram menor incidência de diarreia na fase após o desmame e um melhor peso ao abate do que os suínos que não receberam antibióticos ou aditivos. Como os resultados de desempenho foram semelhantes entre os suínos que receberam antibióticos ou aditivos, podemos concluir que o ácido benzoico associado aos óleos essenciais funciona com substituto aos antibióticos.

No experimento 2, foi avaliada a inclusão de produtos de leveduras e óleos essenciais em diferentes protocolos preventivos via ração. Os aditivos foram usados substituindo ou reduzindo a inclusão de antibióticos, em dietas da fase de crescimento e terminação. Não houve queda no desenvolvimento dos suínos quando os aditivos foram incluídos reduzindo a inclusão dos antibióticos. O uso dos aditivos aumentou o número de espécies bacterianas presentes nas fezes dos suínos. Já a inclusão de doses de antibióticos acima das recomendadas reduziu este número, além de gerar aumento de custo na produção dos suínos sem gerar vantagens no desenvolvimento. Desta forma podemos concluir que é possível manter a produção de suínos com um menor uso de antibióticos.




**Estratégias nutricionais com aditivos, com o objetivo de minimizar o uso de antibióticos via ração na produção suína, têm sido alvo de diversos estudos. Dois experimentos foram conduzidos para avaliar a substituição ou redução de antibióticos por aditivos. O uso de ácido benzoico e óleos essenciais do desmame ao abate melhorou a saúde e o peso dos suínos suplementados. O uso de leveduras e óleos essenciais pode substituir ou reduzir o uso de antibióticos sem prejudicar o desenvolvimento dos suínos.**

*Tese de doutorado em Zootecnia na UFLA, defendida em 19/05/2021.*

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**SUMMARY**

# CHAPTER 1

## 1 INTRODUCTION

In the search for the maximum growth performance, it is of fundamental importance to act on the critical points in each pig production phase, mainly in the first days of accommodation in the nursery phase. This period is decisive, since the weight gain in the first week of nursery phase is directly related to the age at slaughter, and the greater the gain in this period, the less time to reach the slaughter weight.

Several authors report negative effects on the intestinal integrity of piglets after the conditions imposed by weaning, and it is common to observe a decrease in villus height and a reduction in enzyme activity.

In addition, pork meat stands out on the world market as the most consumed animal protein. However, in addition to meeting this growing demand, producers must seek productive efficiency and greater profitability. On the other hand, pressure from more demanding consumers has led to a discussion about the decrease in the use of growth-promoting antibiotics in pig farming, which requires further research for alternative additives. This problem requires the development of nutritional solutions that can reduce or even solve the withdrawal of antibiotics and, thus, improve the intestinal health parameters of the animals. Nutritional solutions can study both new additives and new possibilities for preventive protocols that use these additives efficiently.

Among the additives that can be used as alternatives to antibiotics, benzoic acid, yeast products and essential oils have been evaluated. Studies show that benzoic acid has antibacterial action and positive effects on the performance of newly weaned piglets. Yeast products can improve feed intake and modulate the intestinal immune response, as it contains functional compounds such as mannan oligosaccharides and beta-glucans. The active components of yeast can promote the improvement of physiological parameters related to intestinal health, provide greater resistance and less colonization by pathogenic bacteria. Essential oils extracted from plants have also been explored in pig feed, mainly due to their antimicrobial, antioxidant, and immunomodulatory properties, and thus being an alternative to the use of antibiotics. We understand that there is a potential synergism in these association that can contribute beneficially to the growth performance of pigs and reduce the total use of antibiotics (growth promoter and prophylactic forms).

Finally, the use of natural alternatives has been increasingly consolidated as an alternative in the feeding of pigs for promoting better results in their performance, as well as benefits in their health status. This study can make a solid contribution to decision making in favor of these alternatives in pig production. In addition, it brings new concepts and possibilities of preventive protocols that can be applied to reduce or replace antibiotics. The new concept of food safety highlights the need for a better understanding of the nature, mode of action and reflexes of the use of these compounds in animal performance.

# 2 LITERATURE REVIEW

## 2.1 Use of antibiotics in swine production

In-feed antibiotics (ATB) have been widely used to increase growth rates and prevent diseases for pigs (VAN BOECKEL et al., 2015). Subtherapeutic doses of ATB are used as feed additives to promote growth performance, improving average daily gain (ADG) and feed efficiency (FE), and are known as antibiotic growth promoters (AGP) (GASKINS; COLLIER; ANDERSON, 2002). By the 1960s, industrialized animal production had emerged and role ATB in this story of systems proliferation was initially that of a universal supporting to control disease pressure, increase yields, reduce labor costs, and contain economic risks for producers. However, with farm sizes and productivity rising rapidly, their supporting function was soon seen as essential for the smooth running of food production (KIRCHHELLE, 2018). Several studies between 1950 and 1985 found that ATB were the most effective agents for reducing mortality and morbidity (CROMWELL, 2002).

Globally, agricultural ATB use likely exceeds human consumption (VAN BOECKEL et al., 2015). The ATB consumption patterns in agriculture vary across regions and countries in the developing world, and even ATB that have been banned in other countries, including the developed countries, are still being used in most developing countries (MOYANE; JIDEANI; AIYEGORO, 2013; ADEBOWALE et al., 2016). China has been registered as the world's leading producer and consumer of both animals and human ATB (TANG et al., 2016). The classes of drugs that are more widely used in agriculture at the global level, which are of growing scientific concern with regards to their potential adverse effects and risk management steps, include the tetracyclines, aminoglycosides, β-lactams, lincosamides, macrolides, pleuromutilins, and sulphonamides (DE BRIYNE et al., 2014; BAYNES et al.,

2016). These ATB have the same mode of actions or belong to the same general classes as those used for humans, a situation that demands the judicious use of these drugs in animal farming (GELBAND et al., 2015). In 2010, Brazil was de third country with the largest shares of global antimicrobial consumption in food animal production, and the pig production the animal production that the most consumed ATB (VAN BOECKEL et al., 2015).

Antibiotics added to the diet can improve reproductive performance, conception rate, litter size, and farrowing rate in sows (HAYS et al., 1978; MYERS; SPEER, 1973; MESSERSMITH et al., 1966; RUIZ et al., 1968). In addition, weaning period is often associated with development of intestinal malfunction and diarrhea duo to many stress factors (HU et al., 2013; RHOUMA et al., 2017). Antibiotics are often included in the diets for weaned pigs to control post-weaning diarrhea and optimize growth performance (VERSTEGEN; WILLIAMS, 2002). Cromwell (1991; 2002) observed that the effect of ATB on ADG and FE was greatest for young pigs and declined as pigs approach market weight. Cardinal et al. (2021), through a meta-analysis, found with the use of ATB an increase in post-weaning ADG but not for growing and finishing pigs. In the growing-finishing phase, the beneficial effects of ATB on body weight gain are accompanied by increased FE, improved appetite, and a superior general appearance of the hair coat and skin (LI, 2017). Cromwell (2002) estimated the net economic benefit of AGP use from post-weaning through the finishing phase of production per market hog and, of this total, he estimated that savings from improvements in FE and ADG represented 47 and 42% of the benefits from ATB, respectively. Cardinal et al. (2021) reported AGP withdrawal in the post-weaning phase increased feed costs by US$ 0,86 per animal and in growing-finishing phase the increase was US$ 3,11. Thus, pigs fed AGP diets have a better performance than pigs did not feed AGP and the withdrawal of AGP increases feed costs.

However, the use of AGP in swine production may pose a risk to public health through the transfer of bacterial resistance to humans (ALBERNAZ-GONÇALVES; OLMOS; HÖTZEL, 2021; KIRCHHELLE, 2018; VAN BOECKEL et al., 2017). Resistance is an inherent side effect associated with the overuse, abuse, or substantial use of ATB (MANYI-LOH et al., 2018). Antimicrobial resistance transmission can occur through direct contact with contaminated people, animals, and food, or through the environment, via animal waste containing resistant bacteria that may contaminate soil and water (WOOLHOUSE et al., 2015; HE et al., 2020). Bacteria have the capability to transfer resistance genes between strains of the same species and between different species, because the fact that the ATB

resistance genes are located on elements that can be immobilized (CHANG et al., 2015). The transmission of these resistance genes is termed horizontal gene transfer, usually by mutation, or lateral gene transfer, and it does occur via transformation, conjugation, and transduction processes (LANDECKER, 2016). Antibiotic residues can also induce the selection of resistant bacteria in the environment (HONG et al., 2020; SABRI et al., 2020), owing to the fact that most of these ATB are not fully metabolized but released into the environment as waste products (MANYI-LOH et al., 2018). Cogliani, Goosens and Greko (2011) pointed out that the low concentrations of these ATB in the environment bring about random and spontaneous mutagenesis.

Due to the possible contribution of in-feed ATB to the development of antibiotic-resistant strains of bacteria, there is an ongoing interest to minimize or completely eliminate the inclusion of ATB in livestock diets in many parts of the world (LUSK; NORWOOD; PRUITT, 2006). Although, there is an international mobilization to encourage measures of prudent use of ATB in livestock, in low- and middle-income countries sales of veterinary ATB are unregulated (VAN BOECKEL et al., 2019). Based on the European Union (EU) experience, a ban on the usage of in-feed ATB is usually accompanied by serious production consequences, such as an increase in weaning age (HEO et al., 2013) and a reduction in the number of piglets weaned per sow per year (HAYES; JENSEN; FABIOSA, 2002). Denmark indicated that there was a reduced performance and increased morbidity in nursery pigs, which emphasized the therapeutic use of AB in farms (STEIN, 2002). Therefore, there is a growing demand to find safe alternatives to antibiotic usage (GONG et al., 2014). Despite this, the age, housing system, and moving stress can had as much of an effect on the shedding of resistant bacteria than did the presence of ATB in the diet (CROMWELL, 2002). Thus, the total ban of in-feed ATB from animals can not completely eliminate the antibiotic resistance problem.

As a global health emergency, conforming international recommendations for the prudent use of ATB is a stance that countries that depend on the export of livestock products must adopt (ALBERNAZ-GONÇALVES; OLMOS; HÖTZEL, 2021). The PAN-BR is a plan put forward by the Brazilian Health Ministry together with other government entities to adapt to the practices of rational use of ATB in the coming years (BRASIL, 2018). Pressure from foreign markets may require Brazilian health agencies and the animal production chain to rapidly adapt to the international scenario of restriction of the use of ATB (WORLD BANK

GROUP, 2019). These changes require rethinking the current production systems that rely on high ATB use.

Some studies show how important it is to call for urgent and concerted action in all countries, which is needed to limit the overuse and abuse of antimicrobials in food animal production (ALBERNAZ-GONÇALVES; OLMOS; HÖTZEL, 2021; DUTRA et al., 2021; VAN BOECKEL et al., 2015; LAXMINARAYAN et al., 2013). These actions should include implementation of a publicly funded international surveil-lance network of antimicrobial consumption in food animals in countries undergoing rapid intensification in the livestock sector, and collaboration with veterinary drug manufacturers and animal feed producers to cross-validate estimates of consumption with sales data. However, so far, knowledge of the threat has failed to translate into effective international plans for ATB reductions (KIRCHHELLE, 2018). In addition, implementation of an international agenda to harmonize regulatory frameworks among countries (ALBERNAZ-GONÇALVES; OLMOS; HÖTZEL, 2021), and the ultimate phasing out of antimicrobial use for growth promotion, based on the successful experience in the EU and the new biological (XU et al., 2021; ALLEN et al., 2013) and economic (TEILLANT; LAXMINARAYAN, 2015) evidence challenging the purported benefits of antimicrobial use in food animal production. Brazilian farmers are still reluctant to adopt in use ATB practice changes, rooted in an unchallenged dependency on ATB (ALBERNAZ-GONÇALVES; OLMOS; HÖTZEL, 2021). Furthermore, ATB use did not always reflect the health condition or the real therapeutic needs, easily leading to overuse (DUTRA et al., 2021).

## 2.2 Strategic use of additives

To overcome the increased rate of mortality and morbidity because of AGP ban, several alternatives such as feed additives and dietary interventions have been proposed (HEO et al., 2013). By eliminating antibiotic growth promoters from diets fed to newly weaned pigs, disease problems may be increased, and growth performance may be reduced. In contrast, removal of antibiotic growth promoters from diets fed to growing-finishing pigs does not always result in increased disease problems (WIERUP, 2001). Several feed additives may be effective in regulating intestinal environments and improving pig growth performance if diets without AGP are fed (LIU et al., 2018).

### 2.2.1 Benzoic acid

In the face of speculation about the prohibition on AGP and in the search for additives to replace them efficiently, studies were directed to evaluate the application of organic acids (OA) in the diet of pigs and minimize the problems existing in the post-weaning phase (PAPADOPOULOS et al., 2017; LONG et al., 2018; HAN et al., 2020). Organic acids were initially studied as a substance to preserve the integrity of stored grains, as their ability to reduce pH inhibits the growth of fungi and bacteria (PARTANEN; MROZ, 1999).

The OA most used in animal nutrition, as performance-enhancing additives, are short-chain acids, which contain up to seven carbons. They are the acids: benzoic, formic, acetic, propionic, butyric, lactic and sorbic (PARTANEN et al., 2002). It has been shown that most of OA has a pKa (the pH at which the acid is half dissociated) between 3 and 5 and possess antimicrobial activities (KHAN; IQBAL, 2015). The acidifying action of organic acids occurs through the reduction of pH in dissociated form, and its bactericidal or bacteriostatic action because of this acidification or through its diffusion through the bacterial cell membrane in its non-dissociated form (MODINA et al., 2019). The efficiency of the acid will depend on its exposure time, its concentration, the type of acid, the composition of the basal diet and the age of the animal (COSTA et al., 2013). Among the pigs, an improvement in performance is found, attributed to the antimicrobial effect and the beneficial effect on digestibility and nutrient absorption (FREITAS; LOPES, 2006). However, the response to supplementation is more pronounced in weaned piglets due to the immature digestive physiology of these animals (SUIRYANRAYNA; RAMANA, 2015).

As an organic acidifier, benzoic acid ($C_7H_6O_2$, BA) is a colorless crystalline solid, it stands out as the simplest aromatic carboxylic acid (SIM; ROBERTSON; GOODWIN, 1955). It is mainly absorbed and transported in small intestines via the monocarboxylic acid transporter 1. The absorption rate for the jejunum is higher than those for the duodenum and ileum, which is consistent with the distribution this transporter 1 (BRIDGES et al., 1970).

It has a relatively high dissociation constant (pKa = 4.21), being considered a weak acid, with low solubility and being found in the stomach in the non-dissociated form. This condition allows BA to passively diffuse through the bacterial cell membrane and, since its internal environment is characterized by a pH greater than the acid's pKa, it dissociates. As a result, the bacterium undergoes a decrease in its internal pH, needing to activate a resistance mechanism that reacts to this type of cellular stress. In addition, the process can cause

bacterial death by exhaustion due to the excessive activation of the $Na^+/K^+$ pump to control the internal pH (LUCKSTADT; MELLOR, 2010).

Benzoic acid and sodium benzoate are commonly used as preservatives in the food industry (DEL OLMO; CALZADA; NUÑEZ, 2015; KNARREBORG et al., 2002). In the non-dissociated form, sodium benzoate passes easily through the cell wall of fungi, releasing protons that acidify the medium (KUNG; STOKES; LIN, 2003). Inhibiting the development of fungi is a way to enhance the availability of nutrients in the feed.

Benzoic acid stands out among the acidifiers used to control infections caused by microorganisms in the urinary tract of sows (KLUGE; BROZ; EDER, 2010). According to Mroz (2005), BA increases the digestibility of amino acids and nitrogen, increases urinary acidity, and reduces the emission of ammonia from waste. As the main route of excretion of BA is urinary, it promotes the reduction of bacterial proliferation in the bladder.

Benzoic acid has also demonstrated a wide range of results in improving growth performance (DIAO et al., 2016; ZHAI et al., 2017; PAPATSIROS et al., 2011), maintaining the balance of intestinal microbiota (DIAO et al., 2014), improving intestinal morphology (CHEN et al., 2017; HALAS et al., 2010; GHELER et al., 2009) and digestibility of nutrients (DIAO et al., 2013; KIARIE et al., 2018).

Some results indicating increased nutrient digestibility and digestive enzyme activity with 0.5% BA supplementation that may explain the improved growth performance of supplemented pigs, whether nursery pigs (DIAO et al., 2016; KIARIE et al., 2018) or growing pigs (BÜHLER et al., 2009; SAUER et al., 2009). Studies also show that BA has antibacterial activity (DIAO et al., 2014; HALAS et al., 2010) with positive effects on the growth performance of weaned piglets (ZHAI et al., 2017) and effects that can be seen until the finishing phase (SILVEIRA et al., 2018). Dietary supplementation of BA decreases the pH value of digesta in ileum, cecum, and colon of piglets (DIAO et al., 2014), which can be a critical factor for the selective suppression of the pathogens and the improved biodiversity of microbiota in the gut (HALAS et al., 2010; TORRALLARDONA; BADIOLA; BROZ, 2007).

Considering that the jejunum is the fundamental tissue for the digestion, absorption, and transport of nutrients, it is particularly important that its structure is complete (LOW, 1979). In the BA supplementation, the improved intestinal morphology can be related to the upregulation of mucosal glucagon-like peptide 2 (GLP-2) or insulin-like growth factor I (IGF-1) and activities of superoxide dismutase and glutathione peroxidase (DIAO et al., 2016; MAO et al., 2019). These should be the possible reasons that BA improves gut-epithelial

integrity. Some results suggest that BA administration can improve nonspecific barrier mechanisms in the gut (MAO et al., 2019). A study in which weaned pigs were utilized showed that dietary BA supplementation could stimulate the expressions of ZO-1 and occludin in the jejunal mucosa (CHEN et al., 2017) Thus, BA is an efficient additive to be used in the post-weaning period.

### 2.2.2 Essential oils

Among the strategies that improve the growth performance of pigs and reduce the use of in-feed ATB, the researchers also found in essential oils (EO) beneficial components to the intestinal health of these animals (ZHAI et al., 2018; OMONIJO et al., 2018).

Essential oils are secondary metabolites of plants. These compounds are lipophilic and highly volatile (PUVAČA et al., 2013), formed in cells or groups of cells found in different parts of the plant such as: leaves, stems, flowers, fruits, and roots (FRANZ; NOVAK, 2009). They are commonly isolated from plants by a steam distillation process, and their biological actions make it possible to use them in medicinal, hygiene, perfumery, and cosmetics areas (ADORJAN; BUCHBAUER, 2010).

These compounds work as a defense mechanism of the plant against physiological stress due to lack of water, changes in the climate, protection against pathogens, among others. Thus, the composition of metabolic constituents and the bioactivity vary according to the type of plant, type of soil, climatic conditions during its development, extraction method and harvest time (TUREK; STINTZING, 2013; VIGAN, 2010).

The components belong to two distinct groups of biosynthetic origin: the main one, composed of terpenes and terpenoids, and the group with the lowest frequency of aromatic compounds. These groups, for example, consist of alcohols, ethers, esters, aldehydes, and phenols (PICHERSKY; NOEL; DUDAREVA, 2006). They can consist of variable mixtures of 20 to 60 components in different concentrations, being characterized by two or three main components in high concentrations (BAKKALI et al., 2008; MIGUEL, 2010). These major components are isolated and widely evaluated. Therefore, the EO of a plant have an active principle that corresponds to the compound present in greater quantity in its composition. As an example, oregano (*Origanum vulgare*) has antioxidant and antimicrobial effects attributed to carvacrol (30%) and thymol (23%), which are its two main phenols (BAKKALI et al., 2008). Besides thymol and carvacrol, ρ-cymene was found as another dominant component of

oregano (BOUHADDOUDA; AOUADI; LABIOD, 2016), it could facilitate the transport of carvacrol across the cytoplasmic membrane despite is not an effective antimicrobial agent by itself (OKE et al., 2009). Another example is thyme oil (*Thymus vulgaris*), which contains thymol, carvacrol, ρ-cymene, and γ-terpene in large quantities, giving it excellent antioxidant activity (DANDLEN et al., 2010). They provide broad spectrum antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, and yeast (ABBASZADEH et al., 2014). Cinnamaldehyde, found in cinnamon in 90%, and eugenol, found in cloves in 80% are also extensively studied. Although the main effect of oil is the responsibility of its primary active principle, it is believed that the secondary principles act as its enhancers, with a synergistic effect (KAMEL, 2000).

Before so many variations and interactions of the different EO, knowing the mode of action and understanding the effect of this additive on the performance, development, and health of the intestinal mucosa of piglets is especially important. One of the most active effects of EO is their antimicrobial activity (OMONIJO et al., 2018). Essential oils have a lipophilic characteristic and low molecular weight, which allows them to cross the bacterial plasma membrane, release ions ($H^+$) and depolarize the cell. There is a collapse of the proton pump and high energy expenditure. Loss of potassium can also cause problems, since it plays important roles in the activation of a number of cytoplasmatic enzymes, in maintaining osmotic pressure and in the regulation of intracellular pH. Generally speaking, bacteria can use ionic pumps to counter these effects and cell death does not always happen, but large amounts of energy are needed for this function and bacterial growth is compromised (ULTEE; BENNIK; MOEZELAAR, 2002). Damage to the cell wall and plasma membrane can result in lysis and leakage of macromolecules (OUSSALAH; CAILLET; LACROIX, 2006). Although carvacrol and thymol have several target sites in bacterial cells, the biosynthetic machinery of bacterial cell walls is their main target site (YAP et al., 2014). The leakage also can happen through cytoplasm coagulation and membrane protein destruction (ULTEE; BENNIK; MOEZELAAR, 2002). The decrease in energy for production of toxins can also impair the development of this bacterium and, consequently, decrease its pathogenicity and the occurrence of diarrhea in piglets (BAKKALI et al., 2008). Most studies investigating this action agree that, in general, it is stronger against Gram-positive bacteria than Gram-negative bacteria (BRENES; ROURA, 2010). Therefore, the primary mechanism of action for thymol, carvacrol, eugenol and cinnamaldehyde is related to their effects on the cytoplasmic membranes and energy metabolism (OMONIJO et al., 2018).

Some EO could influence the gut microflora selectively (ZHANG et al., 2020; LI et al., 2018). A blend of carvacrol, cinnamaldehyde, and capsicum oleoresin increased the population of *Lactobacilli* and the ratio of *Lactobacilli* to *Enterobacteria* in the jejunum (MANZANILLA et al., 2006) and cecum (CASTILLO et al., 2006) of early-weaned piglets using qPCR method. This agrees with the *in vitro* study results that cinnamaldehyde was highly inhibitory for coliform bacteria and *E. coli* while it hardly inhibited the growth of *Lactobacilli* (MICHIELS et al., 2008).

Several *in vivo* studies indicated that EO increased the *Lactobacillus* group and decreased *E. coli* or total coliforms in piglets (OMONIJO et al., 2018). Li et al. (2012) evaluated the possibility of modulating the microbiota of weaned piglets, supplementing them with an EO product, whose active compounds were thymol and cinnamaldehyde. They were able to observe a marked reduction in the count of *E. coli* and an increase in the count of *Lactobacillus*, indicating this modulation. Thus, the animals showed a decrease in the incidence of diarrhea, an increase in feed consumption and an improvement in feed conversion ratio (FCR). With the improvement of intestinal health, a greater availability of essential nutrients for absorption and an improvement in the growth performance can be promoted.

The antimicrobial action of the components of some EO is favorable for the maintenance of the epithelium, since, by reducing the incidence of pathogenic bacteria or by inhibiting it, consequently they decrease the production of toxins. In this way, villi compromised by stress, low consumption of solid diets and insufficient energy, will not suffer from an aggravation caused by the imbalance between commensal and pathogenic bacteria (MANZANILLA et al., 2009; WINDISCH; ROHRER; SCHEDLE, 2009).

However, the literature is equivocal regarding the use of EOs as feed additives in relation to gut morphology. There are reports that show increased, unchanged as well as reduced villus length and crypt depth in the jejunum and colon for broilers and piglets fed EOs (ZHANG et al., 2020; ZOU et al., 2016a; MANZANILLA et al., 2009; 2006; 2004). When assessing the intestinal morphology of weaned piglets, Liu et al. (2013) were able to observe a greater height of villi in the jejunum and ileum. The stimulation of EO on intestinal morphology can be a direct way of determining improvement on the growth performance and productivity.

Oxidative stress represents an important chemical mechanism that leads to biological damage, which in turn can affect growth performance and health in pigs, especially in modern

high performance swine production systems (LAURIDSEN, 2019). Pigs are frequently exposed to several stressors including weaning, malnutrition, disease challenge, heat stress, in-feed mycotoxin contaminations, transportation, and overcrowding (WEI et al., 2017; AKBARIN et al., 2016; LYKKESFELDT; SVENDSEN, 2007)

Synthetic antioxidants such as ethoxyquin, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used as effective feed additives in pig diets in order to increase the stability of feed and protect nutrients from oxidation (REISHE; LILLARD; EITENMILLER, 1998). However, such synthetic antioxidants do not have biological effects *in vivo*, and their toxicological safety is also a concern (SHAHIDI, 2000).

This has driven the search for natural compounds that could not only replace synthetic antioxidants in pig feed, but also provide additional zootechnical benefits (YANG et al., 2015). It is well known that EO and plant extracts have anti-oxidative effects (BASCHIERI et al., 2017) and they have been used successfully in animal diets (LIU et al., 2017; WEI et al., 2017; ZOU et al., 2016b). According to Brenes and Roura (2010), the antioxidant activity occurs due to the chemical structure of the phenolic compounds existing in plants. The phenolic groups act as hydrogen donors for the peroxide radicals, produced during the first step of lipid oxidation, and neutralize these radicals, preventing the extension of this process.

Zeng et al. (2015) indicated that pigs fed an EO diet had higher levels of albumin, immunoglobulin(Ig) A, IgG, total antioxidant capacity and better fecal score when compared to pigs fed control diets. A *in vivo* pig study has shown that dietary supplementation with mixture of carvacrol and thymol decreased the weaning associated intestinal oxidative stress through decreasing tumor necrosis factor alpha (TNF-α) (WEI et al., 2017).

Another important action of EO is the ability to stimulate the production of saliva, gastric juice, and pancreatic juice (JANG et al., 2004), associated with the presence of tannins or pungent substances in plant extracts (MELLOR, 2000).

According to Jones (2002), the increase in gastric juice production provides a reduction in stomach pH, providing ideal conditions for the activity of digestive enzymes in the stomach, such as pepsin. Wang, Li and Bourne (1998) observed that capsaicin, an active ingredient in pepper, increased the production of saliva and the secretion of sucrase and maltase in rats, in addition to the action of cinnamaldehyde, the active principle of cinnamon, on the enzymes sucrase and maltase. However, Muhl and Liebert (2007) did not observe improved nutrient digestibility and enhanced pancreatic and duodenal activity of trypsin and

amylase in weaned piglets fed diets containing a phytogenic product having carvacrol, thymol and tannins as key constituents.

Stimulation of enzyme activity will result in good digestion and absorption of nutrients (LI et al., 2012; AHMED et al., 2013) and a modulation of the intestinal microbiota, also interconnecting with positive changes in the morphology of the intestinal epithelium (PLATEL; SRINIVASAN, 2004).

### 2.2.3 Yeast products

Among of natural alternatives to AGP that produce similar effects on performance and overall animal health are yeast cell wall products derived from *Saccharomyces cerevisiae* (BROADWAY; CARROLL; SANCHEZ, 2015). *S. cerevisiae* is a species of yeast that has been used for centuries for a variety of processes including, but not limited to, brewing and bread-making. Some time, yeast and yeast cell wall components have been utilized as a supplement in the swine feed (VAN DER PEET-SCHWERING et al., 2007). Yeast products (YP) are often fed to pigs as either a live yeast, as yeast cell wall, or as a combination of the two (XIONG et al., 2015). One of the major components of yeast and yeast cell wall products are polysaccharides such as α-D-glucan and β-D-glucan (KOGAN; KOCHER, 2007). The cell wall components also can be provided purified such as mannan oligosaccharide (MOS) and β-glucans (ROBINSON; ERASMUS, 2009).

B-glucans are a heterogeneous group of natural polysaccharides, composed of glucose monomers linked together by β-glycosidic bonds. They are found as important structural elements of the cell wall or as a way of storing energy in bacteria, filamentous fungi, yeasts, algae, and plants, being absent in vertebrate and invertebrate tissues (STIER; EBBESKOTTE; GRUENWALD, 2014).

There are several ways in which YP elicit their effects, including enhancing intestinal barrier function, altering bacterial populations within the gastrointestinal (GI) tract, and through providing beneficial substrates for host bacteria (KOGAN; KOCHER, 2007). Altering GI tract permeability by enhancing nutrient absorption, while limiting pathogenic bacteria translocation and release of toxins systemically, can greatly benefit the host through reducing inflammation. This is often a result of altering mucin production (BONTEMPO et al., 2006), tight junctions between epithelial cells (PAN et al., 2017), and crypt and villi characteristics within the GI tract (DI GIANCAMILLO et al, 2007). The supplementation of

YP may improve energy availability during an immune challenge, which may be beneficial to allow for a more rapid resolution (BURDICK SANCHEZ; BROADWAY; CARROLL, 2021).

These polysaccharides not only interact directly with immune cells but are also able to bind bacteria to prevent attachment and colonization of pathogens in the gastrointestinal tract (RUIZ-HERRERA, 1992). In addition to pathogen mitigation (KOGAN et al., 1989), yeast cell wall components may possess antioxidant (KOGAN et al., 2005) and antitumor properties (KHALIKOVA et al., 2005). From an animal health and immunity perspective, yeast cell wall components from *Saccharomyces cerevisiae* have been reported to promote the release of cytokines from macrophages (VETVICKA, 2011; MAJTÁ et al., 2005), and may be involved with modulation of immune cells in many species (MEDZHITOV; JANEWAY, 2000). β-glucans can be recognized as pathogen associated molecular patterns by the innate immune system (BAERT et al., 2015), stimulating phagocytosis and the production of reactive oxygen species (ROS's) in phagocytes for microbial death.

When administered orally, β-glucans tend to interact with defense cells present in Peyer's plaques in addition to lymphocytes present in the intraepithelial space of the intestine (VOLMAN et al. 2008). Studies have shown that a very low amount of β-glucan administered orally is absorbed, suggesting that its primary action is in defense cells present in the intestine and the secondary results are the effect of this interaction (SANDVIK et al., 2007).

Glucans exert adjuvant activities through their capability to bind to specific surface carbohydrate receptors, such as CR-3 and Dectin-1, expressed on the monocyte-macrophage cell lineage and other antigen-presenting immunocompetent cells, as dendritic cells. The attachment of glucan molecules to these receptors results in the activation of a cascade of pathways that subsequently increase the production of proinflammatory cytokines and chemokines inducing antigen presentation and cellular co-stimulation, which leads to enhancing of both humoral and cellular immunity (VETVICKA; VANNUCI; SIMA, 2014).

Li et al. (2006) found that diets supplemented with glucan partially down-regulated the synthesis of proinflammatory cytokines TNF-α and interleukin (IL)-6 modulating immunity but also regulating metabolism of nutrients. In addition, glucan up-regulated anti-inflammatory cytokine IL-10 inhibiting T cell proliferation and functional differentiation including secretion of Th1 and Th2 cytokines, which simultaneously exhibits signal transduction pathway of NF-κB (CLARKE et al., 1998).

The production of the various types of immunoglobulins can be affected by glucans, with lower doses favoring IgA (SAUERWEIN; SCHMITZ; HISS, 2007). Specific response to immunization was not clear with some reporting lowering effects of anthropic rhinitis vaccine (HAHN et al., 2006) and some reporting enhanced effects of immunization with ovalbumin (LI et al., 2006). Besides β-glucans can significantly lower the cortisol and TNF-α levels after lipopolysaccharide challenge (VETVICKA; OLIVEIRA, 2014).

β-glucans are originally used for their immunomodulatory potential, which can improve intestinal health by increasing mucosal barrier functions in order to contribute to the growth of animals (EWASCHUK et al., 2012), since the necessary nutrients to growth are digested and absorbed by the body through the intestinal mucosa. These polysaccharides may also have an influence on the composition of the intestinal microbiota (MURPHY et al., 2012), which not only helps in digestion, but can directly and indirectly support the immunological activities of the intestine-associated lymphoid tissue (GALT), representing the largest immune organ in mammals (VETVICKA; VANNUCCI; SIMA, 2014).

Mannan oligosaccharides are often referred to as a potential alternative for AGP (HALAS; NOCHTA, 2012). In the yeast cell wall, MOS are present in complex molecules that are linked to a protein moiety (LIPKE; OVALLE, 1998). There are two main locations of MOS in the cell wall of *Saccharomyces cerevisiae* (STEWART; RUSSELL, 1998), either attached to cell wall proteins (LESAGE; BUSSEY, 2006) or as elements of larger α-D-mannose polysaccharides, which consist of α-(1,2)- and α-(1,3)- D-mannose branches (from 1 to 5 ring structures in length), which are attached to extended α-(1,6)-D-mannose chains (VINOGRADOV; PETERSEN; BOCK, 1998).

This specific combination of various functionalities involves MOS-protein conjugates and highly hydrophilic, but variable, ‘brush like’ structures that have attachment ability for various receptors within the digestive tract (MANSOUR; LEVITZ, 2003), and on the surface of bacterial membranes (WELLENS et al., 2008). This allows MOS to effectively bind the thread like fimbriae on pathogenic bacteria (OYOFO et al., 1989), rendering them unable to attach to the gut wall, preventing their stabilization and subsequent colonization and multiplication to disease-causing levels. Since mucosal surfaces including gastro-intestinal, nasal, and broncho-alveolar organs represent the largest part of the animal body permanently exposed to the attack of pathogens and toxins, stimulation of the Common Mucosal Immune System (CMIS) represents a crucial task of animal health protection (TLASKALOVÁ-HOGENOVÁ et al., 2002).

MOS-protein conjugates are involved in interactions with the animal's immune system and can enhance immune system activity (CHE et al., 2011; WISMAR et al., 2010) and it is thought that they play a role in antioxidant and antimutagenic defenses (KRIZKOVA et al., 2006).

A well-balanced microflora within the digestive tract is important for normal functioning (ANDERSON et al., 2000). Intestinal bacteria require attachment for colonization in intestinal mucosa enabling their harmful effects. In Gram-negative bacteria like *Salmonella sp* and *E. coli*, the attachment is carried out with type-1 fimbriae, which contain mannose-specific lectins named FimH. MOS reduce the attachment of Gram-negative bacteria with intestinal mucosa by binding with bacterial FimH of type-1 fimbriae. In this way, the bacteria move through the intestine without allowing colonization (BAURHOO; PHILLIP; RUIZ-FERIA., 2009; HOOGE et al., 2003; FERNANDEZ; HINTON; VAN GILS, 2002). The microbiota exists in a dynamic state and increasing the number of any bacterial specie may result in the decrease of another specie. Some studies show that the reduction in the number of pathogenic bacteria in response to dietary MOS supplementation was indeed associated with an increase of beneficial flora, particularly *Lactobacillus* (LIU et al., 2008; REKIEL et al., 2007; WHITE et al., 2002). Sims et al. (2004) found in turkey that dietary MOS supported the growth of *Enterococcus*. These bacteria produce not only short chain fatty acids (SCFA) but also bacteriocin and enterocin and thus enhance the competition and development of beneficial flora (LEROY; FOULQUIE-MORENO; DE VUYST, 2003). Other results also confirm the positive effect of MOS, since it is reported to reduce the ammonia concentration in the gut (JUSKIEWICZ; ZDUNCZYK; JANKOWSKI, 2003).

There are several hypotheses on the beneficial effect of MOS on intestinal morphology, but not all of them were proven in swine (HALAS; NOCHTA, 2012). The reduction in the *Enterobacteria* population (CASTILLO et al., 2008) and increase in beneficial flora (LIU et al., 2008; REKIEL et al., 2007) enhance the SCFA production in the intestine, which positively affects the recovery of the epithelia. A number of studies reported that dietary MOS supplementation increases the lactic acid and volatile fatty acid production in the hind gut (POEIKHAMPHA; BUNCHASAK, 2011; REKIEL et al., 2007). In particular, butyric acid has anti-inflammatory property, alleviating the hypersensitive reaction of the gut wall associated with the post-weaning period. In pigs, a prompt recovery of the intestinal mucosal cells was promoted by MOS (CAINE; SAUER; HE, 2001).

Researchers has shown increased production of enzymes such as maltase, leucine aminopeptidase, and alkaline phosphatase in the brush border lining the intestines of animals supplemented with MOS (FERKET, 2002; YANG et al., 2008). To protect the villi and intestinal surface, the gut produces more mucus from specific cells called goblet cells. In general, the number of goblet cells is an indicator of mucus production, and researchers have found that goblet cell numbers were increased with MOS. The importance of such changes for animal health is still being debated by scientists (SPRING et al., 2015).

Regarding performance, two meta-analyses, involving a total of 123 comparisons (MIGUEL; RODRIGUEZ-ZAS; PETTIGREW, 2004; ROSEN, 2006) concluded that performance was better in piglets fed MOS-supplemented feed. The data indicated that pig which were particularly challenged during the weaning transition phase, responded particularly well to MOS supplements. Positive performance effects with MOS have been reported in later production phases but appear to be smaller in magnitude compared to responses seen in very young animals (MIGUEL; RODRIGUEZ-ZAS; PETTIGREW, 2004). From overall metanalysis of ten years of pig trial data, Rosen (2006) showed that 73% of trials showed better weight gain and 68% improved FCR. In addition to the economic benefits resulting from the maintenance of gut health and support of the defense mechanisms, the use of medication can be reduced with the use of dietary MOS supplementation that enables healthier and safe, and therefore more desirable pork production (HALAS; NOCHTA, 2012).

## 2.3 Immunity of intestinal mucosa

The intestine plays an important role in the digestion and absorption of ingested foods and in the elimination of undigested food and microbial products (BACKHED et al., 2015). The functional integrity of the intestinal mucosal epithelial cells depends on the coordinated regulation of the mucus layer, narrow intercellular junction, epithelial cells, and the innate and adaptive immune response of the host (OKUMURA; TAKEDA, 2016).

The intestinal immune system has the difficult task of protecting us from pathogenic insults by limiting the inflammatory responses against the resident commensal microbiota. Thus, it is perhaps not surprising that the intestine contains the greatest number and diversity of immune cells in the body (AGACE; MCCOY, 2017). In addition to the existence of the microbiota, the immune response in the intestinal mucosa differs because the intestine has direct communication with the external environment. So, the immune system in this region is

critical to maintaining tolerance to both these proteins and to the microbiota. Its structure must maintain a balance between the effector response against the pathogens and a regulatory response that inhibits inflammation (GROSS; SALAME; JUNG, 2015).

The epithelial surface of the gastrointestinal tract constitutes a physical barrier, thus providing a first layer of defense against infection (PETERSON; ARTIS, 2014). A second defense medium is found adjacent to the epithelial cells: mucus. The mucus layer consists of a complex web of mucin and antimicrobial proteins that cover the epithelial surfaces of the gastrointestinal tract, thus preventing microorganisms from reaching the epithelial cells (SICARD et al., 2017). A third defense layer is provided by the numerous immune cells that reside in the intestine, either in organized structures, such as Peyer's patches and mesenteric lymph nodes, or scattered throughout the intestinal epithelium and lamina propria (PEREZ-LOPEZ et al., 2016).

Intestinal epithelial cells (IECs) maintain a fundamental immunoregulatory function that influences the development and homeostasis of intestinal mucosal immune cells (ZHANG; HORNEF; DUPONT, 2015). The intestinal epithelium is the largest of the mucosal surfaces of the body, and although most of the cells that delimit the intestinal lumen are enterocytes, which are adapted for metabolic and digestive function, the diversity of functions that the intestinal epithelium plays is reflected by the presence of specialized IECs (PETERSON; ARTIS, 2014), which are extremely important for the initiation and regulation of mucosal inflammation (KAGNOFF, 2014).

Secretory IECs, including enteroendocrine cells, goblet cells, and Paneth cells, are specialized to maintain the digestive or barrier function of the epithelium (PETERSON; ARTIS, 2014). The enteroendocrine cells are responsible for the secretion of numerous hormonal regulators of the digestive function (GRIBBLE; REIMANN, 2016). The luminal secretion of antimicrobial mucins and peptides by goblet cells and Paneth cells, respectively, establishes a physical and biochemical barrier to microbial contact with the epithelial surface and the underlying immune cells (ZHANG; HORNEF; DUPONT, 2015).

The IECs express pattern-recognition receptors (PRRs), which allow them to act as dynamic sensors of the microbial environment and as active participants in the targeting of mucosal immune cell responses (KAGNOFF, 2014), producing mediators that recruit, activate, and condition cells of the immune system (MOWAT; AGACE, 2014). In addition, the activation of these receptors results in proliferation and cellular restitution and generates protection against apoptosis (ABREU, 2010).

Toll-type receptor family members, NOD-type receptors (NLRs), and RIG-like receptors (RLRs) provide distinct pathways for the recognition of pathogenesis-associated microbial ligands or endogenous signals (PETERSON; ARTIS, 2014). Pathogen associated molecular patterns (PAMPs) represent conserved structural motifs produced by commensal pathogenic microorganisms. PRRs recognize PAMPs by initiating transcriptional and non-transcriptional cellular responses (ABBAS; LICHTMAN; PILLAI, 2015).

The proximity of IECs to an abundance of luminal microbial signals requires specialized mechanisms to maintain altered or hyporesponsive PRR signaling in response to commensal-dependent stimuli (THAISS et al., 2016). For this, IECs express negative regulators of proinflammatory signaling dependent on PRRs (ABREU, 2010).

Furthermore, innate immune pathways should differentiate between signals derived from commensal and pathogenic microorganisms for scheduling an appropriate inflammatory response (PETERSON; ARTIS, 2014). The polarized nature of the intestinal epithelium allows the anatomical segregation of PRRs. *In vitro* and *in vivo* models demonstrate the differential responsiveness of IECs to apical or basolateral stimulation with multiple TLR ligands (ABREU, 2010; LEE et al., 2006). Analysis of human IECs indicated that TLR5 is expressed only on the basolateral surface, where it can trigger the production of cytokines and chemokines, such as the chemokine ligand IL-8 and CC 20 (CCL20), in response to flagellin. In this way, luminal flagellin can activate TLR5 only after lesion of the epithelial barrier (ABREU, 2010). Receptors of the NLR family, such as NAIP and IPAF-1, also bind to the flagellin and are expressed in the cytoplasm of the IECs, so that the pathogen invades the cell to activate the receptor response (ABBAS; LICHTMAN; PILLAI, 2015).

The innate immunity in the gastrointestinal tract, in addition to being composed of the physical and chemical barrier of the epithelium, is formed by the phagocytic immune cells (PETERSON; ARTIS, 2014). The innate immune cells, besides being sentinels that detect microorganisms or damaged cells, in this case, are also responsible for intestinal homeostasis (ABBAS; LICHTMAN; PILLAI, 2015). The IECs exert their influence on cellular immune and adaptive humoral responses through a continuous dialogue with antigen-presenting mononuclear phagocytes (CEROVIC et al., 2014).

The mononuclear phagocytes consist of macrophages and dendritic cells (DCs), both of which have been implicated in the uptake and presentation of antigens in the intestine (MOWAT; AGACE, 2014). They have distinct but complementary roles in maintaining intestinal homeostasis and immune defense (GROSS; SALAME; JUNG, 2015). They are

scattered throughout the lamina propria, and are located in associated lymphoid tissues, including Peyer's patches and isolated lymphoid follicles (GROSS; SALAME; JUNG, 2015).

The macrophages are the most abundant mononuclear phagocytes in the lamina propria of the intestine (FARACHE et al., 2013; MOWAT; AGACE, 2014). They have several important functions in intestinal homeostasis, including phagocytosis and degradation of microorganisms and dead tissue cells, as well as the production of mediators that drive epithelial cell renewal (GROSS; SALAME; JUNG, 2015). They also 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 T-cell regulatory (Treg) FoxP3+ cells in the mucosa (HADIS et al., 2011). The production of IL-1β by resident macrophages in response to the microbiota may have an analogous ability to maintain the activity of Th17 cells in the small intestine at steady state (SHAW et al., 2012). These properties are quite specific to the gut and, unlike many other tissue macrophages, those of the small intestine and colon mucosa are derived by the continuous replacement of blood monocytes that differentiate locally under the control of the mucosal environment (BAIN et al., 2014).

Besides to its role in maintaining intestinal immune homeostasis, macrophages of the intestine also contribute critically to the healing of epithelial wounds. Macrophages associated with the crypts of the colon have been reported to assist, after tissue injury, the proliferation and survival of epithelial progenitor cells. Tissue repair was positively regulating the expression of IL-3 and IL-4, while inhibiting the secretion of TNF and interferon-γ (IFN-γ) in the lamina propria (FARACHE et al., 2013). Macrophages also appear to be able to influence the permeability of the epithelial barrier via secretion of IL-6 and nitric oxide (NO), thus potentially increasing pathogen invasion (GROSS; SALAME; JUNG, 2015).

In all parts of the intestine, macrophages are found relatively close to the epithelial surface, but this appears to be more prominent in the colon. Despite this, no marked functional difference was reported between small bowel and colon macrophages (PETERSON; ARTIS, 2014). Thus, similar processes seem to control the development and functions of macrophages throughout the intestine, although they have different markers and receptors (MOWAT; AGACE, 2014).

The dendritic cells are specialized in T-cell communication, inhibiting autoreactivity through tolerogenic mechanisms, and activating T-cell immunity in response to danger (GROSS; SALAME; JUNG, 2015). CD103+ DCs, present in the lamina propria and

associated with the intestinal epithelium of the villi, promote the surveillance of the luminal environment. They detect foreign and inflammatory signals, acquire, and present antigens and interact with T-cells migrating to secondary lymphoid organs (BEKIARIS; PERSSON; AGACE, 2014). Because T-cell activation is restricted to lymphoid tissues, intestinal DCs are required to migrate from the lamina propria to the mesenteric lymph nodes (MLNs), or within the Peyer's patches, to the T-cell sites (JANG et al., 2006).

Although generally 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 (ROTHKÖTTER; KIRCHHOFF; PABST, 1994). It is believed that eosinophils are important for tissue repair both at steady state and during inflammation in the small and large intestines (LAMPINEN et al., 2013).

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

Recently discovered functions of goblet cells confirm their central role in the innate immune response. Goblet cells from the small intestine detect luminal contents at the time of mucus secretion, may absorb intraluminal antigenic material and, in a manner not fully understood, provide these antigens to the DC in the lamina propria (PELASEYED et al., 2014). These routes for the transport of antigens are called antigenic passages associated with goblet cells (GAPs). It is not fully understood whether the mechanism of absorption of goblet cells is mediated by vesicles or if the whole cytoplasm is filled. Under normal conditions, the ability of goblet cells of the colon to form GAPs is disrupted and they do not collect luminal material (JOHANSSON; HANSSON, 2016).

The immune system produces mediators that affect the function of goblet cells (BIRCHENOUGH et al., 2015). For example, helper T (Th2) cells drive goblet cell hyperplasia and mucus hypersecretion by IL-4, IL-5, IL-9, and IL-13 production. In addition to the importance of Th2 cytokines in the regulation of goblet cell function, Th17-associated

cytokine IL-22 can also be associated in the regulation of goblet cell differentiation and mucin expression (TURNER; STOCKINGER; HELMBY, 2013).

Members of the TLR family are key mediators of communication between microorganisms and hosts and promote homeostasis, with important implications for mucus formation. Bacterial metabolites, such as short chain fatty acids (SCFAs), influence the protective function of the epithelium (SICARD et al., 2017). Butyrate is an SCFA which is especially important as it is a preferred source of energy for colon epithelial cells, and decreased absorption of butyrate is associated with both intestinal inflammation and carcinogenesis. Goblet cells use butyrate as an important source of energy for the production of MUC2 when other energy sources are limited, such as the distal colon (JOHANSSON; HANSSON, 2016). Other functions of SCFAs include regulation of intestinal blood flow, production, enterocyte growth and proliferation, and intestinal immune responses (PAN; YU, 2014).

Different regions of the gut are continually exposed to distinct environmental factors, and this is likely to markedly affect the composition of the immune cells. As the composition and density of the microbiota vary greatly between different intestinal sites, these differences may also have specific effects on the formation of regional variation in immune cell populations (MOWAT; AGACE, 2014).

## 2.4 Microbiota and its relationship with immunity and growth performance

The swine intestinal microbiota is considered an important “organ” with a crucial role to play in nutrient processing and the harvesting of ingested energy (FOUHSE; ZIJLSTRA; WILLING, 2016). The gut microbiota is known to provide other beneficial functions for the host including the re-cycling of bile salts, production of vitamin K, and the production of exogenous alkaline phosphatases (GILLILAND; SPECK, 1977; RAMOTAR et al., 1984). The microbiota is also an essential stimulus that results in the maturation of the animal’s gut immune system (BIK, 2009; POSTLER; GHOSH, 2017).

It is now known that the relationship between innate immunity and the microbiome extends well beyond the careful balance between tolerance to commensal microorganisms and pathogen immunity (KABAT; SRINIVASAN; MALOY, 2014). The microbiota integrates into the physiology of the organism and influences multiple aspects of homeostasis through its effects on the innate immune system (PFEIFFER; VIRGIN, 2016).

The impact of microorganisms on IECs extends well beyond the classical immunological functions of these cells (THAISS et al., 2016). Commensal bacteria play an important role in the metabolism of IECs (GOTO; KIYONO, 2012), such as the production of SCFAs or induction of the production of intestinal hormones. SCFAs affect both oxygen consumption and hypoxia inducible factor (HIF) mediated "strengthening" of the epithelial barrier (KELLY et al., 2015). The bacterial metabolite promotes the barrier function through the pregnane X receptor (PXR), also known as NR1I2 (VENKATESH et al., 2014), and increases secretion of glucagon-like peptide 1 (GLP-1) by enteroendocrine cells (CHIMEREL et al., 2014). There is also a role for acetate, derived from *Bifidobacterium*, in promoting anti-apoptotic responses in IECs (FUKUDA et al., 2011). In addition, peptidoglycans derived from the cell wall of commensal bacteria activate the cytoplasmic NOD1 receptors in the IECs and induce the secretion of β-defensin 3 and CCL20, which boosts the formation of isolated lymphoid follicles (KABAT; SRINIVASAN; MALOY, 2014). Both, the IECs integrate microbial signals both in the orchestration of the host-microbiome interface, which consists of the production of mucus and epithelial antimicrobial proteins, and in the dynamic adjustment of cellular metabolism (THAISS et al., 2016).

Shaped by genetic and environmental factors, especially diet, intestinal microbial diversity in pigs is also affected by the breed (PAJARILLO et al., 2014; YANG et al. 2014), the animal's growth stage (KIM et al. 2015; HAN et al., 2018), and the intestinal segment of pigs (LOOFT et al., 2014; KIM; ISAACSON, 2015).

There also are a possible link between the intestinal microbiota and growth performance, mainly feed efficiency (FE) in pigs (TAN et al., 2018; MCCORMACK et al., 2017; XIAO et al., 2017; YANG et al., 2016). The gut microbiota has been shown to be involved in regulating the energy-harvesting efficiency and improving the energy-harvesting capacity of the host (TURNBAUGH et al. 2006). These factors are associated with body weight gain (KIM et al. 2015; KIM; ISAACSON 2015; LOOFT et al. 2012, 2014). Increased knowledge of the community structure and functional capacity of the gut microbiota helps to reveal relationships between microbial functions and the host's physiology and metabolism. Furthermore, taxonomy and functional capacity of fecal microbiota were determined in low and high FCR broilers (SINGH et al. 2014).

Finally, multiple previous studies in pigs found that the gut microbiome of pigs with higher FE contained more SCFA producing bacteria (TAN et al., 2017; YANG et al., 2017; QUAN et al., 2018), suggesting an important role of enzymatic activities of the microbiome in

regulating FE. However, the exact mechanisms remain to be defined. Because of the large contribution of SCFA by cellulytic bacteria (DIERICK et al., 1989), these particular bacteria may increase the efficiency of feed utilization of the hosts. Specifically for FE, the gut microbiome has a clear role in regulating weight gain in humans (LEY et al., 2006).

Cecum microbiota in high FE pigs have slightly higher richness and evenness than low FE pigs and some predominant species of the high FE pigs have a greater ability to utilize dietary polysaccharides and proteins (QUAN et al., 2020). Higher bacterial diversity in the GIT, including that of pigs, is generally seen as favorable. Han et al. (2017) found also higher diversity within the feces of heavier compared to lighter pigs at 9 weeks of age. In particular, bacteria from the genus *Prevotella* might impair the establishment of a more effective nutrition harvesting microbiota because the interaction between them and other beneficial microbes (QUAN et al., 2020).

There is also a different microbial community structure in the fecal microbiota of pigs with different FE. Quan et al. (2019) detected 24 OTUs that can serve as potential biomarkers to improve swine FE. The high-FE pigs had a greater abundance of OTUs in the families *Lachnospiraceae* and *Prevotellaceae* and in the genera Escherichia-Shigella and *Streptococcus* compared to low-FE pigs. The fecal microbiota in high FE pigs, besides to having a greater capacity to degrade nutrients, may have a greater abundance of microbes to promote intestinal health.

Han et al. (2017) observed a lower fecal relative abundance of *Bacteroides* and *Anaerotruncus*, two genera that contain pathogenic species (YEKANI et al., 2020) in the heavier pigs. The results showed a concomitant reduction in genes related to the nucleotide-binding oligomerization domain-like receptor (NLR) signaling pathway, which is associated with induction of a host immune response, which is energy-demanding. Hence, reduced activation of this bacterial pathway, possibly due to lower pathogen levels, could explain the improved growth in these animals. Yang et al. (2016) also found potential pathogens (*E. coli* and pathogenic species of *Clostridium*) to be less abundant in the ileum and caecum of leaner pigs, a phenotype which is more desirable.

In general, the growth- and FE-associated taxa are mostly associated with nutrient processing and energy harvest for the host, but some are related to anti-inflammatory effects and improved gut health (GARDINER; METZLER-ZEBELI; LAWLOR, 2020). Low-grade gut mucosal inflammation caused by bacterial abundance or metabolites may, therefore, be

sufficient to redirect energy in low-FE pigs that is otherwise available for growth in high-FE pigs (FROSALI et al., 2015).

## 3 FINAL CONSIDERATIONS

Over time, the use of in-feed antibiotics modified animal production and consolidated results of performance, health, and economic viability of the activity. The idea that there is a single additive or combination that is as efficient as antibiotics is still far away. However, feeding pigs with subtherapeutic doses or overuse of antibiotics in the long term leads to the development of antimicrobial resistance, which is putting public health at risk. Currently, there is great interest in the production of pigs without antibiotics. Therefore, to avoid the negative effects of removing antibiotic growth promoters from pig diets, changes in management and nutritional strategies are necessary.

In this scenario, our objective was to evaluate the reduction, or even the replacement of the in-feed antibiotic with associated additives, not only the use as antibiotic growth promoters, but also prophylactic forms. After all, this is already a reality in several pig productions in the world.

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# CHAPTER 2

## ARTICLE 1 - BENZOIC ACID AND ESSENTIAL OILS MODIFY THE CECUM MICROBIOTA COMPOSITION IN WEANED PIGLETS AND IMPROVE GROWTH PERFORMANCE IN FINISHING PIGS

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## RESUMO

O ácido benzoico (AB) e os óleos essenciais (OE) possuem propriedades antimicrobianas e podem ser usados em situações de restrição a antibióticos. A combinação desses aditivos tem se consolidado cada vez mais como uma alternativa para promover melhor desempenho, bem como benefícios para a saúde dos suínos. Nossa hipótese é que o uso de AB e OE pode minimizar as perdas de desempenho devido à remoção dos antibióticos nas dietas e alterar a saúde intestinal de leitões desmamados. O objetivo deste estudo foi avaliar os efeitos da associação do AB e dos OE na resposta imune celular e humoral sérica; morfologia, expressão gênica e capacidade antioxidante na mucosa jejunal; microbiota do conteúdo cecal, no pós-desmame e desempenho dos suínos da creche à terminação. Cento e vinte suínos machos castrados foram desmamados (6,40 ± 0,53 kg) e distribuídos em três tratamentos em um delineamento de blocos casualizados (10 repetições): dieta basal sem aditivos (CN), dieta basal com antibióticos (CP) e dieta basal com 0,3% de BA e OE (AB+OE). No tratamento CP foram usados 200 ppm de colistina na creche, 5 e 10 ppm de enramicina na fase de crescimento e terminação, respectivamente. Os componentes ativos do AB+OE foram o ácido benzoico e uma mistura de óleos essenciais que incluiu timol, 2-metoxifenol, eugenol, piperina e curcumina. Os suínos foram pesados aos 0, 42, 84 e 132 dias de avaliação. A incidência de diarreia foi avaliada diariamente na fase de creche. Nos dias 1, 3 e 9, amostras de sangue foram coletadas. No dia 9, esses animais foram eutanasiados para coleta de amostras de jejuno e conteúdo cecal. Na fase de creche, os leitões do grupo CP apresentaram maior peso e ganho médio diário (GPD) e menor conversão alimentar (CA) em comparação com o grupo CN, mas semelhantes aos do grupo AB+OE ($P<0,050$). Na fase de crescimento, o tratamento CP resultou em maior peso e consumo médio de ração diário (CMRD), e na fase de terminação o peso do grupo AB+OE foi semelhante ao do grupo CP e maior que o do grupo CN ($P<0,050$). No período total, o peso e o GPD dos grupos CP e AB+OE foram semelhantes e superiores aos do tratamento NC ($P<0,050$). Os leitões AB+OE tiveram a menor incidência de diarreia durante o período de creche ($P<0,050$). O uso de AB+OE reduziu a contagem de leucócitos totais e neutrófilos ($P<0,050$). Quando comparada com o grupo CP, a suplementação com AB+OE aumentou significativamente os níveis de glutationa e diminuiu a atividade da superóxido dismutase na mucosa jejunal ($P<0,050$). Nas análises do microbioma, observou-se que os grupos AB+OE e CP apresentaram microbiota cecal semelhantes quando comparados com os leitões CN. Um aumento significativo no número de unidades taxonômicas operacionais (OTUs) foi observado no grupo AB+OE. Os suínos CP e AB+OE apresentaram menor abundância de *Bacteroidetes* do que o grupo CN ($P<0,001$). Em conclusão, a suplementação com AB+EO reduziu a resposta inflamatória e modificou o microbioma cecal no período pós-desmame, resultando em melhora no desempenho de suínos em terminação.


**Palavras-chave:** Aditivos. Suínos. Diarreia. Saúde intestinal. Microbioma.

**Benzoic acid and essential oils modify the cecum microbiota composition in weaned piglets and improve growth performance in finishing pigs**

Maíra Resende[a], Rhuan Filipe Chaves[a], Ricardo Miranda Garcia[a], Jéssica Aparecida Barbosa[a], Athos Silveira Marques[a], Lilian Ribeiro Rezende[a], Ana Paula Peconick[a], Cesar Augusto Pospissil Garbossa[b], Dany Mesa[c], Claudia Cassimira Silva[d], Vitor Barbosa Fascina[d], Francine Taniguchi Falleiros Dias[d], Vinícius de Souza Cantarelli[a*]

[a]Animal Science Department, Federal University of Lavras, Lavras, Brazil
[b]Department of Animal Nutrition and Production, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Pirassununga, Brazil
[c]Department of Biosciences, Federal University of Paraná, Campus Palotina, PR, Brazil
[d]DSM Nutritional Products, Animal Nutrition and Health, São Paulo, Brazil

[*]Corresponding author
E-mail address: vinicius@ufla.br

**ABSTRACT:** The objective of this study was to evaluate the effects of benzoic acid (BA) and essential oils (EO) on cellular and humoral immune response, morphology, gene expression, antioxidant capacity in the jejunal mucosa, and cecal content microbiota in weaned piglets and growth performance from the nursery to finishing phase. One hundred and twenty barrows were weaned and assigned to three treatments in a randomized block design: basal diet without additives (NC), basal diet with antibiotics (PC), and basal diet with 0.3% BA and EO (BA+EO). The pigs were weighed 0, 42, 84, and 132 days into the experiment. The incidence of diarrhea was assessed daily in the nursery phase. On days 1, 3, and 9, blood samples were collected. On day 9, these animals were euthanized to collect jejunal samples and cecal content. In the nursery phase, the PC piglets showed a greater body weight (BW) and average daily gain (ADG) and a lower feed conversion ratio (FCR) compared to the NC group, but similar levels to the BA+EO group ($P < 0.050$). In the growing phase, the PC treatment resulted in a greater BW and average daily feed intake (ADFI), and in the finishing phase the BW of the BA+EO group was similar to that of the PC group and greater than that of the NC group ($P < 0.050$). In the total period, the BW and ADG of the PC and BA+EO pigs were similar and higher than those for the NC treatment ($P < 0.050$). The BA+EO pigs had the lowest incidence of diarrhea during the nursery period ($P < 0.050$). The use of BA+EO reduced the counts of total white blood cells (WBC) and neutrophils ($P < 0.050$). When compared with the PC group, BA+EO supplementation significantly increased the glutathione (GSH) levels and decreased the superoxide dismutase (SOD) activity in the jejunal mucosa ($P < 0.050$). In the microbiome analyses, it was observed that the BA+EO and PC groups had similar cecal microbiota when compared to the NC piglets. A significant increase in the number of operational taxonomic units (OTUs) was observed in the BA+EO group. The PC and BA+EO pigs showed a lower abundance of *Bacteroidetes* than the NC pigs ($P < 0.001$). In conclusion, supplementation with BA +EO reduced the inflammatory response and modified the cecal microbiome in the post-weaning period, resulting in an improvement in the growth performance of finishing pigs.

**Keywords**: Additive, diarrhea, gut health, microbiome, swine.

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## 1. Introduction

In the post-weaning period, piglets are vulnerable to the consequences of stress caused by nutritional, environmental, social, and immunological changes (Moeser et al., 2017; Pluske, 2016; Smith et al., 2010). The negative impacts on the growth performance of weaned piglets relate to damage to the intestinal villi, with a subsequent decrease in the digestibility of nutrients (Hu et al., 2013), favoring an increased incidence of diarrhea (Rhouma et al., 2017).

To maintain the balance of the microbiota in the weaned piglet gastrointestinal tract, the main nutritional additives used in the last six decades have been antibiotics that, in subtherapeutic doses, act as growth promoters, decreasing mortality rates and increasing productive efficiency (Verstegen and Williams, 2002). However, the use of these products in animal nutrition has been increasing restricted in recent years, since it is attributed to increased resistance of pathogenic bacteria to antibiotic treatments in humans and animals (Tang et al., 2017).

To minimize animal damage and economic problems at this stage, as well as to seek alternatives to antibiotics, BA and EO have been evaluated (Heo et al., 2013; Kiarie et al., 2018; Zhang et al., 2020). Studies show that BA has antibacterial activity (Diao et al., 2014; Halas et al., 2010) with positive effects on the growth performance of weaned piglets (Diao et al., 2016; Zhai et al., 2017) and effects that can be seen until the finishing phase (Silveira et al., 2018). Modulation of the microbiota and improved growth performance are also effects observed in piglets supplemented with EO (Li et al., 2018). Finally, the combination of these additives has been increasingly consolidated as an alternative to promote better growth performance, as well as health status benefits (Diao et al, 2015, Zhai et al., 2020; Zhang et al., 2016). There are several studies demonstrating the additive effects of some essential oils and organic acids (Hulánková and Bořilová, 2011; Souza et al., 2009; Zhou et al., 2007). Recent studies have clearly shown *in vivo* efficacy of such synergistic dietary strategies in pigs (Balasubramanian et al., 2016; Walia et al., 2017; Silva Júnior et al, 2020). Some of the mechanisms underlying this potential synergism between some EO and BA are still not clear. However, it is well-known that phenols in essential oil can change the structure and functions of bacterial cell membranes, leading to increased membrane permeability to BA (Pu et al, 2018).

This assessment can make a solid contribution to deciding in favor of these alternatives within pig production. The new concept of food safety highlights the need for a better understanding of the nature, modes of action, and impacts of the use of these additives

on animal performance (Kiarie et al., 2016). To unravel these distinct effects on microbiota, traditional performance trials in association with microbiome technology provide new opportunities (Kim and Isaacson, 2015).

We hypothesized that the use of BA and EO can minimize growth performance losses due to the removal of antibiotics in nursery, growing, and finishing diets and change the intestinal health of weaned piglets. Therefore, the objective of this study was to evaluate the effects of BA+EO on cellular and humoral immune response, morphology, gene expression analyses, antioxidant capacity in the jejunal mucosa, and cecal content microbiota in weaned piglets and growth performance from the nursery to finishing phase.

## 2. Materials and methods

The experimental design and procedures were approved by the Ethics Committee on Animal Use of the Federal University of Lavras under Protocol 080/18. The benzoic acid and essential oils (VevoWin®) were provided by DSM (São Paulo, Brazil).

### *2.1 Animals, experimental design, and housing*

The experiment was conducted in the weaning and growing/finishing facilities at the Department of Animal Science of the Federal University of Lavras, in Lavras, Brazil. A total of 120 weaned barrows were obtained from a commercial pig herd (DanBred sows x PIC 337 sires) with an average initial body weight of 6.40 ± 0.53 kg (approximately 23 days of age). The pigs were randomly allocated in a randomized block design, with three treatments: negative control (NC), using a basal diet without additives; positive control (PC), with colistin sulphate (200 ppm) in the nursery diets and 10 and 5 ppm enramycin in the growing and finishing diets, respectively; and 0.3% combination of benzoic acid and essential oils as an alternative additive (BA+EO). The contents of the active components of the alternative additive were 90% benzoic acid and the blend of essential oils included thymol, 2-methoxyphenol, and eugenol, with an estimated total of 10%, and piperine and curcumin, with an estimated total of 3% (VevoWin®). Ten replicates (4 pigs/pen) were used in the nursery phase. At day 9 post-weaning, seven pigs per treatment (heaviest replicates) were removed for euthanasia and sample collection. At day 42 post-weaning (end of nursery phase), one pig (with the closest BW to the group mean BW from each replicate that had three pigs) was removed in order to meet the floor space requirements in growing-finishing phases. Therefore, ten replicates of two pigs per pen were used in the growing-finishing phases.

For the nursery phase (days 0 to 42 of the trial), 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 five-space feeder and nipple drinkers. For the growing/finishing phase (days 43 to 132 of the trial), the pens allowed a floor space of 1.15m² per pig and had a totally compact floor. All piglets were provided with feed and water in a two-space feeder and nipple drinkers.

*2.2 Diets and experimental procedures*

The experimental period of 132 days was divided into eight diets. The basal diet was formulated to meet or exceed the nutritional specifications suggested by the NRC (2012) for pigs from the weaning to finishing phases (Supplementary Table S1). The pigs 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 as a growth promoter. However, for the control of respiratory diseases, the pigs received one dose of tulathromycin (Draxxin®, Zoetis, 100 mg/ml), 0.15 ml per animal, at weaning.

The feed intake and individual body weight were recorded at 0, 42, 84, and 132 days. Based on these data, the ADG, ADFI, and FCR were calculated. For fecal scoring, the feces per animal 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 the nursery phase, the occurrence of diarrhea was calculated as a percentage of the phase days.

*2.3 Sample collections*

On days 1, 3, and 9 of the trial, blood samples were collected from one piglet per treatment from the seven heaviest replicates, totaling 21 animals. The piglets with the closest individual ADG to the group mean ADG were chosen. Blood samples were obtained in heparinized tubes from the anterior vena cava, and the serum was separated and immediately froze at -20°C for further analyses. For the WBC counts, blood samples were obtained in EDTA tubes and the analyses were performed immediately. On day 9 of the trial, all piglets were weighed before slaughter and the same 21 animals selected for blood collections were euthanized by electronarcosis followed by exsanguination.

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 gene expression and redox parameter analyses. Additionally, the caecum digesta was collected aseptically and stored at -80°C for microbiota analyses.

*2.4 Serum levels of cytokines and white blood cell counts*

Serum interleukin (IL)-1β and 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 IL-1β, 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 IL-1β and IL-10 kits were 6 pg/ml and 18.75 pg/ml, respectively. The intra- and inter-assay CV of the IL-1β kit were <8% and <10%, 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, 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 Gene expression analyses*

Total RNA was extracted from the jejunum samples using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The RNA was quantified with a NanoDrop-ND 1000 spectrophotometer

(Thermo Fisher Scientific Inc., Waltham, MA, USA). For verification of the integrity of the total RNA, the 28S and 18S structural bands of rRNA were used as markers in a 1.0% (m/v) agarose gel subjected to electrophoresis. Reverse transcription was performed immediately following the RNA isolation using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in three biological replicates in a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The thermocycle used for the qRT-PCR was 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Gene expression was normalized using two housekeeping genes. The entire real-time PCR experiment included a sample as a negative control (no cDNA) and a calibrator, which was the amplification of a known gene (RPL-32) from an aliquot of the cDNA pool of the samples. The relative amount of each target gene mRNA was determined and recorded as Ct (cycle threshold) values. The relative expression levels were calculated according to the method described by Pfaffl (2001), which is based on Ct values that are corrected for the amplification efficiency for each primer pair. The primer sequences for the target and housekeeping genes (occludin, glucagon-like peptide-2, β-actin, RPL-32) are listed in Supplementary Table S2.

*2.7 Intestinal redox parameters*

For the catalase (CAT) and 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 glutathione-S-transferase (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 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 Cecum 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, 11,673 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 Nucleotide sequence accession number*

All the raw sequences obtained after assembling and filtering were submitted to the NCBI site under accession number BioSample: SAMN15095615.

*2.10 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 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 0.05. The WBC counts were analyzed by repeated-measures analysis of variance, with time as the repeated measure. 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 genders 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. The microbiome figures and their legends were automatically generated by the STAMP program.

**3. Results**

*3.1 Growth performance*

The growth performance results of the animals during the experimental period are presented in Table 1. In the nursery phase, the pigs of the PC treatment showed a greater BW ($P = 0.014$) and ADG ($P = 0.012$) and lower FCR ($P = 0.037$) compared to the pigs from the NC group, but similar levels to the pigs from the BA+EO group. In the growing phase, the pigs of the PC treatment had the greatest BW and ADFI, but in the finishing phase, the BW of the pigs of the BA+EO group was similar to that of the PC group. For the total period, the

BW ($P$ = 0.006) and ADG ($P$ = 0.028) of the PC and BA+EO pigs were similar and greater than those of the pigs from the NC treatment.

*3.2 Incidence of diarrhea*

As shown in Figure 1, during the first seven days of evaluation, the pigs of the BA+EO treatment showed a lower incidence of diarrhea compared to the pigs of the PC group ($P$ = 0.027). From days 8 to 14, the pigs of the BA+EO treatment had the lowest incidence of diarrhea, compared to the pigs of the other treatments ($P$ = 0.005), which remained the case during the total nursery period ($P$ < 0.001). The treatments had no effects from the 22$^{nd}$ to 42$^{nd}$ day period ($P$ = 0.130).

*3.3 Serum levels of inflammatory cytokines and white blood cells*

On both days 3 and 9, the serum IL-1β concentration was not influenced by the treatments ($P$ > 0.050, Figure 2). However, on day 9, the pigs from the PC treatment showed a lower serum IL-10 concentration, compared with the NC pigs but not when compared to the pigs of the BA+ EO treatment ($P$ < 0.050). As shown in Figure 3, the supplementation with BA+EO or the use of antibiotics reduced the counts of total WBC ($P$ = 0.008), with a greater variation in neutrophils ($P$ = 0.003). The pigs of the PC group had the lowest lymphocyte count compared to the pigs of the other treatments ($P$ = 0.013).

*3.4 Jejunal morphology, related gene expression, and jejunal mucosal redox parameters*

The villus height, crypt depth, and villus:crypt ratio data are shown in Table 2. There were no differences between the treatments for jejunum morphology ($P$ > 0.050). When compared to the PC group, BA+EO supplementation significantly increased the GSH activities and decreased the SOD activities in the jejunal mucosa of the weaned piglets ($P$ < 0.050, Table 3). There were no differences in the GST and CAT concentrations in the jejunal mucosa between the three groups. In addition, as shown in Table 4, the antibiotic or BA+EO supplementation did not modify the relative gene expression of occludin and glucagon-like peptide-2 (GLP-2).

*3.5 Cecum microbiota*

The results of the taxonomic classification, by principal components analysis (PCA), showed a marked difference in bacterial communities per treatment (Figure 4). It was observed that samples from the BA+EO and PC groups had similar intestinal microbiota

when compared to the NC group. It was also observed that the treatment was responsible for 82.2% of the changes in the bacterial composition in the evaluation.

In addition, regarding the biodiversity indicators of the bacterial communities, a significant increase in the number of OTUs was observed in the pigs of the BA+EO treatment when compared to the other treatments ($P < 0.050$; Figure 5). There were no differences in the richness index (Chao1) between the groups (data not shown).

We observed a shift in the microbiota composition at the phyla levels (Figure 6). Of the identified phyla, the treatments presented seven phyla with significant differences. The pigs of the PC and BA+EO groups showed a lower abundance of *Bacteroidetes* than the NC group ($P < 0.001$). In addition, the taxonomic classification identified 142 taxa (Supplementary Sheet 1). Using the Kruskal Wallis test, 24 taxa were identified with significantly different abundances between treatments (Table 5). The BA+EO supplementation reduced the abundance of *Prevotella*, *Streptococcus*, and *Megasphaera* (Figure 7).

## 4. Discussion

Benzoic acid and essential oils have been proposed as different nutritional tools to control weaning-associated intestinal dysfunction and subsequent growth failure in pig production (Mao et al., 2019; Omonijo et al., 2018; Zhai et al., 2018). Their distinct actions are complementary (Zhai et al., 2020; Zhou et al., 2007) and well-known beneficial properties in the intestinal mucosa of weaned piglets have been shown when they are used together (Diao et al., 2015; Silva Júnior et al., 2020). Large-scale studies using this combination in the growing-finishing phase are still lacking, as well as studies that show the residual effect in later phases (Silveira et al., 2018).

In our study, as shown by Diao et al. (2015), supplementation with BA+EO improved the FCR during the nursery phase. Previous results indicating increased nutrient digestibility and digestive enzyme activity, as well as a superior microenvironment and better morphology, may explain the improved growth performance of supplemented pigs (Zhang et al., 2016; Silva Júnior et al., 2020). Several studies have found improved nutrient digestibility with supplementation of essential oils (Yan et al., 2010; Maenner et al., 2011; Li et al., 2012), as well as 0.5% BA supplementation (Diao et al., 2013, 2016; Kiarie et al., 2018). As these actions overlap, it is reasonable to save BA with the addition of essential oils, without impairing the growth performance of the piglets. Our study demonstrates this synergism by

using 0.3% of BA+EO, which led to improved FCR. The PC group had greater ADG and BW than the pigs of the NC treatment in this phase, but similar results to the BA+EO group. Antibiotics are often included in the diets of weaned pigs to optimize growth performance (Verstegen and Williams, 2002), and several studies show an improvement in this phase (Long et al., 2018; Kiarie et al., 2018). However, there are growing concerns around the world about indiscriminate use of antibiotics and links to the emergence of antibiotic resistant pathogens (Heo et al., 2013; Tang et al., 2017). For this reason, studies that validate the use of alternative additives are becoming increasingly important.

The BA+EO supplementation in the growing-finishing phase increased the final BW, as did the addition of the antibiotic. Zhai et al. (2017) showed significantly improved growth performance of growing-finishing pigs when supplemented with BA, but this was not found in other studies (Cho et al., 2015; Giannenas et al., 2016; Morel et al., 2019). The effects on growth performance may be associated with an increase in digestibility (Nørgaard et al., 2010), including in the nursery period. The inconsistency in the results found may be explained by the growing-finishing phase having less exacerbated challenges than the nursery phase. In this study, the improvement in final growth performance may be more attributable to the modulatory effect of supplementation with BA+EO promoted in the microbiota and the immune response from the post-weaning period onward, which were probably also maintained during the growing-finishing phase, with their effects on total pig production. However, further studies are warranted to decipher the microbial interactions and the real effect on growth performance and intestinal health (Soler et al., 2018). With increased piglet age, the gut microbiota matures and also becomes more stable (Thompson et al., 2008), wherein less stable and diverse microbiota may be more susceptible to environmental changes, such as in diet, and as a consequence may be more responsive to additive supplementation (Wang et al., 2013).

Considered as one of the factors that most interfere in the growth performance of weaned piglet, post-weaning diarrhea is a commonly-occurring disease (Heo et al., 2013) and characterized by sudden death or diarrhea, dehydration, and growth retardation in surviving piglets (Rhouma et al., 2017). In this study, the BA+EO supplementation decreased the incidence of diarrhea during the nursery phase. Diao et al. (2015) also found this reduction in the incidence of diarrhea, using a combination of BA and thymol, as did Pu et al. (2018), when supplementing piglets with a combination of BA and oregano oil. Several studies have reported that BA supplementation (Chen et al., 2017a; Diao et al., 2014; Halas et al., 2010), as

well as the addition of EO (Li et al., 2018; Ahmed et al., 2013; Zhang et al., 2020), decreases the number of harmful microorganisms. Moreover, there is a synergy in the antimicrobial effect when BA and EO are combined, since the phenolic compounds present in EO cause significant damage to cell membranes, increasing the susceptibility of the bacteria to BA (Omonijo et al., 2018; Pu et al., 2018).

The post-weaning period is characterized by a high level of stress (Moeser et al., 2017), high demand from the immune system (Pluske et al., 1997; Pluske et al., 2018), and dysfunction of the intestinal barrier (Wei et al., 2017). Cytokines play an important role in immune and inflammatory responses and in the regulation of intestinal barrier integrity (Al-Sadi et al., 2009). The overproduction of pro-inflammatory cytokines, such as IL-1β, has a negative impact on gut integrity and epithelial function (Pié et al., 2004). However, in this study, the treatments did not have an impact on the serum concentration of IL-1β and relative gene expression of occludin. Pu et al. (2018) suggested that combined supplementation with BA and oregano oil may improve intestinal integrity by partially suppressing enterotoxigenic *E. coli*-induced proinflammatory cytokine production. Liu et al. (2013) also reported that dietary plant extracts partially counteracted the inflammation induced by an $F18^+$ *E. coli* challenge, consistently with the results of Wang et al. (2011) for LPS-challenged pigs. Different experimental conditions and low health challenges can be the cause of inconsistent results when food additives are used as an alternative to antibiotics (Wang et al., 2018). In the current study, weanling pigs were kept in a clean and sanitary environment, and there was no microbiological challenge. In addition, the results demonstrate that the inclusion of BA+EO or antibiotics can maintain homeostasis in weaned piglets. Occludin is an important integral membrane protein, which has been shown to be a key element in maintaining the structure and function of tight junctions (Lu and Walker, 2001). Tight junctions play a crucial role in intestinal barrier integrity and protect against pathogenic bacteria colonization through paracellular diffusion (Ulluwishewa et al., 2011). Wei et al. (2017) also found no difference in the gene expression of occludin in the jejunal mucosa of weaned piglets supplemented with carvacrol-thymol, despite the increased inflammatory response caused by weaning. Unlike our results, Chen et al. (2017a) found a higher relative expression of occludin in weaned piglets supplemented with BA and Zou et al. (2016) found the same result in pigs receiving oregano essential oil. A lower serum IL-10 concentration was observed in the PC piglets compared with the NC group, but not when compared to the BA+EO group. IL-10 is primarily secreted by T-cells and is considered an important anti-inflammatory cytokine.

Although IL-10 does not appear to affect basal epithelial barrier function, it does appear to be protective against tight junction barrier disturbance (Al-Sadi et al., 2009). The use of BA+EO or antibiotics were able to reduce the total WBC and neutrophil counts in the post-weaning period, reversing the leukocytosis presented by the NC group (Feldman et al., 2000). These results may be beneficial for the growth performance of the piglets, since there is a reduced inflammatory response caused by weaning (Czech et al., 2009; Liu et al., 2013). WBC counts are commonly used to estimate the risk of bacterial infection, and an increase in WBC indicates the presence of systemic inflammation (Gordon-Smith, 2009). The higher concentration of IL-10 observed in the NC group may be associated with the greater WBC count (Ueda et al., 2010).

Weaning stress also induces intestinal oxidative stress (Wang et al., 2008; Wei et al., 2017; Zhu et al, 2012), which contributes to intestinal dysfunction. Although the antioxidant effect of BA (Diao et al., 2016; Mao et al., 2019) and EO (Zeng et al, 2015; Wei et al., 2017) is well-known, this study did not demonstrate that effect. Jiang et al. (2017) suggested that EO supplementation might improve the non-enzymatic reactions of the antioxidant system, attenuate lipid peroxidation, and have the capacity to eliminate free radicals. Moreover, excess BA treatment inhibited the proliferation of IPEC-1 cells, which could be related to the impairment of redox status regulated by the Nrf2 pathway (Gao, 2013). These factors may be linked to our results. The addition of colistin increased the SOD activity, which was not found by Long et al. (2018). However, the GSH concentration was lower in this treatment than in the BA+EO group.

Intestinal morphology is an important factor that reflects pig health and intestinal functionality, as it has a strong relationship with the digestion and absorption of nutrients (Pluske, 2016). However, early weaning has been associated with the destruction of the intestine mucosal integrity of piglets, which could contribute to poor performance (Hu et al., 2013). In this study, the addition of the antibiotic or BA+EO did not interfere in the jejunum morphometry, such as in the relative gene expression of GLP-2. Previous studies have shown that supplementation with 0.5% BA (Chen et al., 2017a; Diao et al., 2016; Halas et al., 2010) or essential oil blends (Zhang et al., 2020; Zou et al., 2016) improved the small intestinal morphology. GLP-2, an enteric peptide produced by enteroendocrine cells, could improve nutrient absorption and gut barrier function, reduce intestinal inflammation, and benefit the performance of the pigs (Connor et al., 2016). GLP-2 reduces the apoptosis of epithelial cells, increases cell proliferation in intestinal mucosa, and promotes growth and regenerative repair

after injury to the intestinal mucosa (Pedersen et al., 2008). The increase in concentration and relative gene expression of GLP-2 found by Diao et al. (2016) may explain the improvement in jejunal morphology and growth performance. The BA concentration used and the experimental conditions in our study were not sufficient to modify the relative gene expression of GLP-2. This result may be associated with the concentrations of IL-1β found, which were also not affected by the treatments.

The intestinal microbiota plays an indispensable role in the health of the host, including in the development of the host's immune response, differentiation of the intestinal epithelium, maintenance of the intestinal mucosal barrier, and absorption and metabolism of nutrients (Postler and Ghosh, 2017). The host intestine and microbiota relationship is versatile and highly susceptible to numerous environmental factors, especially diet (Li et al., 2018). Studies with benzoic acid (Chen et al., 2017a; Diao et al., 2014) and essential oils (Li et al., 2018; Zhang et al., 2020) have demonstrated this relationship. In this study, the use of BA+EO modified the cecum microbiota, as found by Silva Júnior et al. (2020), and increased the number of OTUs. Zhai et al. (2020) evaluated the colon microbiota in nursery pigs supplemented with BA+EO and also found differences using principle coordinate analysis, although the number of identified OTUs remained similar. The compositions of the microbiota of the colon and cecum at the phylum level are very similar to each other (Isaacson and Kim, 2012; Looft et al., 2014) and the constancy of the results found by the studies demonstrate the efficiency of the combination of BA and EO in modulating the microbiota. In humans, gut microbiome diversity is used as a good indicator of a healthy gut (Menni et al., 2017), and less microbial diversity has been observed in people with short bowel syndrome (Lapthorne et al., 2013). The intestinal microbiota also contributes to the immune system, ranging from the normal development of lymphoid tissues to the definition of immune response profiles (Brodin and Davis, 2017). For animals, high bacterial diversity is favorable for overall health and productivity (Hildebrand et al., 2013). In addition, McCormack et al. (2017) suggested a possible link between intestinal microbiota and feed efficiency in pigs, as found in our study. The PC and BA+EO groups showed different cecal microbiota to the NC group in the post-weaning period and a better FCR in that phase. The clinical and physiological changes observed in piglets supplemented with BA+EO, such as the reduction in the incidence of diarrhea and the improvement in microbial diversity, may be related to the improvement in the FCR. The use of antibiotics also modified the cecal microbiota of the piglets evaluated, but did not increase the diversity. Fecal microbiota

analyses have demonstrated that the use of antibiotics can destroy pathogens as well as other members of the intestinal microbiota (Cecilia et al., 2007), thereby reducing diversity (Knecht et al., 2014). Moreover, the microbiota does not immediately return to normal after cessation of antibiotic treatment (Yin et al., 2015).

Consistently with previous research (Chen et al., 2017b; Guevarra et al., 2018; Hu et al., 2016), *Firmicutes* and *Bacteroidetes* phyla were predominant in the cecal microbiota in this study. The ratio of *Firmicutes* to *Bacteroidetes* could be of significant relevance to gut microbiota status. The results suggested that the BA+EO treatment positively changed the structure of the gut microbiota in the weaned piglets (Mariat et al., 2009). In addition, the supplementation with BA+OE decreased the relative abundance of the phylum *Bacteroidetes,* as did the use of antibiotics. Lower abundances of this phylum have been associated with greater weight gain in pigs (Guo et al, 2008a; Guo et al, 2008b), which may explain the improvement in the growth performance of the BA+EO group. The BA+EO supplementation also reduced the abundance of *Prevotella*. Yang et al. (2017) observed a positive association with relative abundance of *Prevotella* in diarrheic pigs, and thus a potential role of *Prevotella* in the community-wide microbial disorder, which is closely related to the pathogenesis of piglet diarrhea. Our study corroborates these results, because the BA+EO group had a lower incidence of diarrhea and a lower abundance of *Prevotella*. As noted by Zhai et al (2020), the combination of these additives shows positive clinical and physiological results for supplemented piglets.

Microbial diversity may be a new indicator of intestinal functions and stability, which are closely related to animal health (Zhang et al., 2020). Furthermore, its analysis offers a promising approach to assess functional status in the interaction between the microbiota and the host intestine.

## 5. Conclusions

In conclusion, supplementation with 0.3% benzoic acid and thymol, eugenol, and piperine essential oils reduced the inflammatory response, modified the microbiome, and increased microbial diversity in cecal content. Moreover, the supplementation reduced the incidence of diarrhea in the nursery phase, resulting in an improvement in the growth performance of the finishing pigs. The results indicate that the combination of benzoic acid and essential oils could be an alternative feed additive to antibiotics.

## Conflict of interest

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

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**Table 1.** Effects of experimental diets on growth performance in piglets from nursery to finishing phase.

| Item[1] | Treatments[2] | | | SEM | P-value[3] |
|---|---|---|---|---|---|
| | NC | PC | BA+EO | | |
| Initial BW, kg | 6.40 | 6.40 | 6.39 | 0.10 | 0.112 |
| Nursery phase, d 0 to 42 | | | | | |
| BW d 42, kg | 25.26$^{b}$ | 27.18$^{a}$ | 25.79$^{ab}$ | 0.39 | 0.014 |
| ADG, g | 447$^{b}$ | 495$^{a}$ | 462$^{ab}$ | 13 | 0.012 |
| ADFI, g | 697 | 755 | 706 | 24 | 0.111 |
| FCR | 1.593$^{a}$ | 1.526$^{b}$ | 1.529$^{b}$ | 0.019 | 0.037 |
| Growing phase, d 43 to 84 | | | | | |
| BW d 84, kg | 62.58$^{b}$ | 69.00$^{a}$ | 65.19$^{b}$ | 1.40 | 0.002 |
| ADG, g | 900$^{b}$ | 995$^{a}$ | 938$^{ab}$ | 25 | 0.011 |
| ADFI, g | 1,665$^{b}$ | 1,898$^{a}$ | 1,716$^{b}$ | 48 | 0.002 |
| FCR | 1.848 | 1.918 | 1.856 | 0.030 | 0.212 |
| Finishing phase, d 85 to 132 | | | | | |
| BW d 132, kg | 110.66$^{b}$ | 115.69$^{a}$ | 114.23$^{a}$ | 1.12 | 0.006 |
| ADG, g | 976 | 966 | 1,022 | 23 | 0.183 |
| ADFI, g | 3,097 | 3,035 | 3,091 | 51 | 0.711 |
| FCR | 3.378 | 3.010 | 3.264 | 0.132 | 0.193 |
| Total period, d 0 to 132 | | | | | |
| ADG, g | 789$^{b}$ | 822$^{a}$ | 815$^{a}$ | 5 | 0.028 |
| ADFI, g | 1,834 | 1,854 | 1,845 | 31 | 0.980 |
| FCR | 2.349 | 2.226 | 2.262 | 0.034 | 0.471 |

[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: negative control; PC: positive control (200 ppm colistin sulphate in nursery diet, 10 and 5 ppm enramycin in growing and finishing diets); BA+EO: supplementation with benzoic acid and essential oils (0.3%).

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

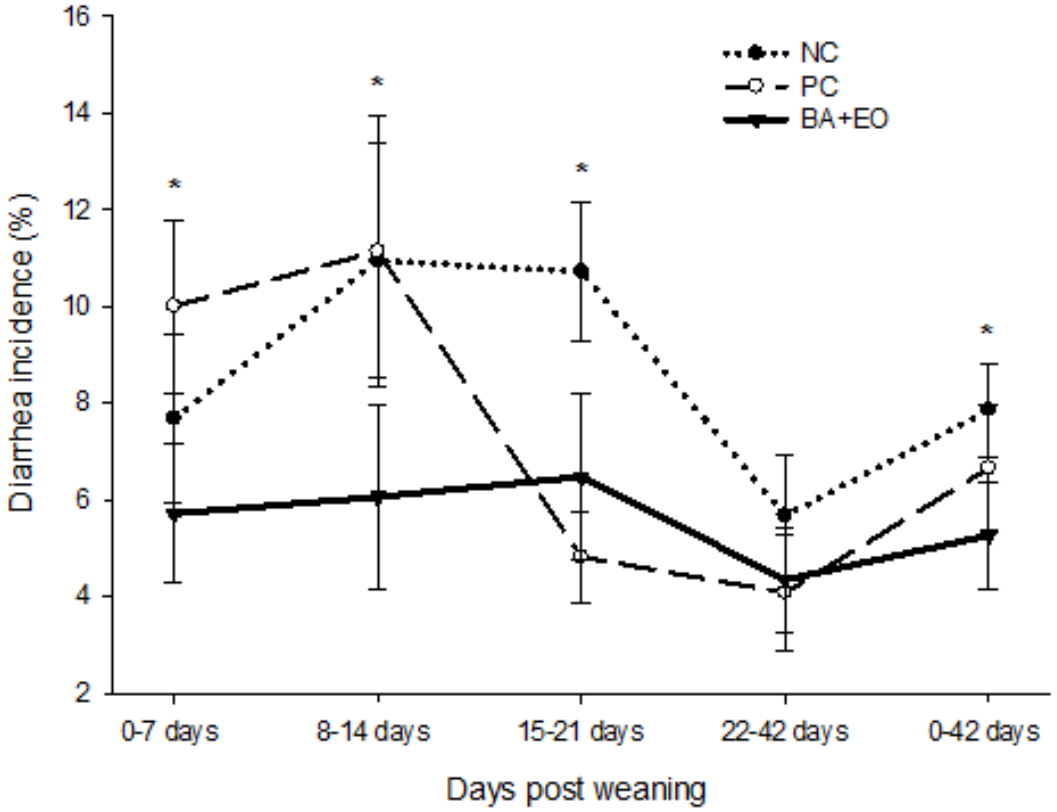


**Figure 1.** Effect of experimental diets on the incidence of diarrhea in piglets in nursery phase. NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%). Data are expressed as means (10 replicates/treatment) and SEM represented by vertical bars. *Significant differences between groups according to binomial analysis, $P < 0.050$.

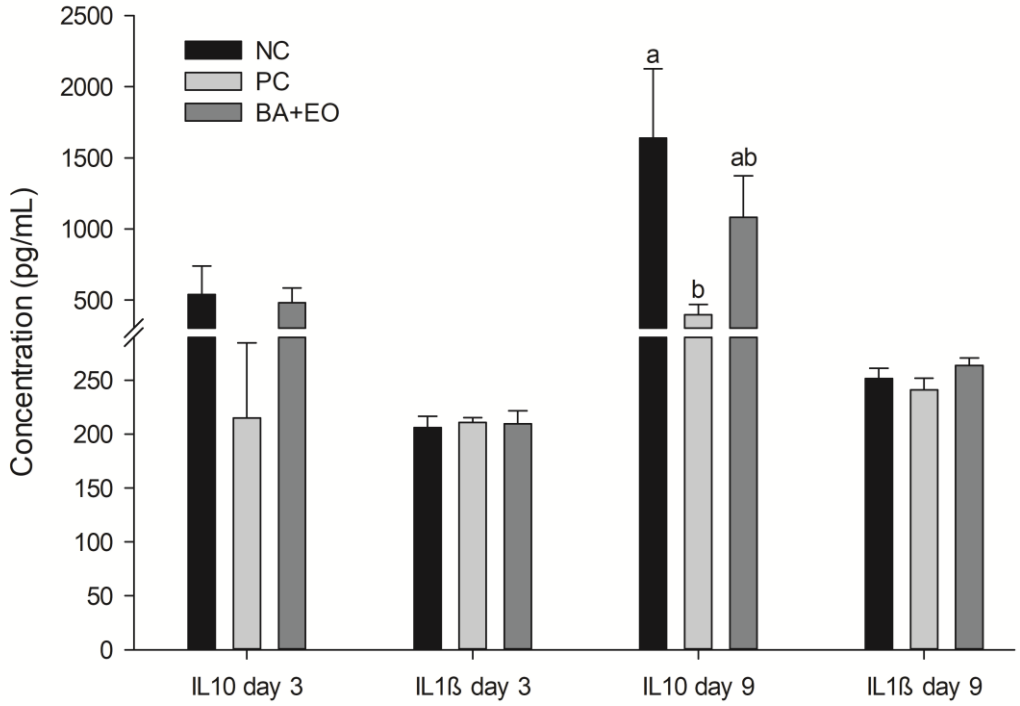


**Figure 2.** Effects of experimental diets on serum levels of interleukin (IL)-1β and IL-10 of piglets on days 3 and 9 post-weaning.

NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%). Data are expressed as means (5 piglets/treatment) and SEM represented by vertical bars. [ab]Significant differences between groups according to Tukey's test, $P < 0.050$.

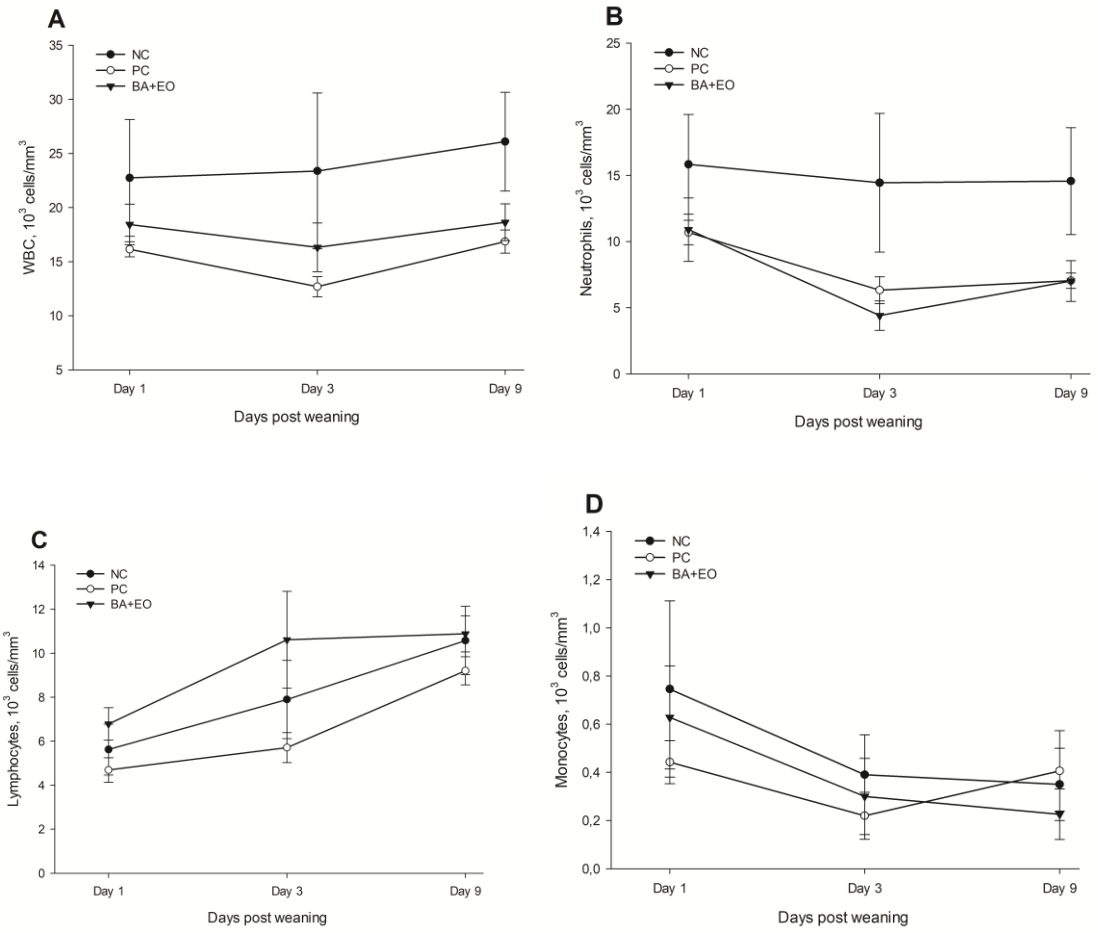


**Figure 3.** Effects of experimental diets on total white blood cells (WBC) (A), neutrophils (B), lymphocytes (C), and monocytes (D) counts of weaned piglets.

NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%). Data are expressed as means (5 piglets/treatment) and SEM represented by vertical bars. *Significant difference from other treatments according to Tukey's test, $P < 0.050$.

**Table 2.** Effects of experimental diets on the jejunal morphology of weaned piglets.

| Item | Treatments[1] | | | SEM[2] | P-value[3] |
|---|---|---|---|---|---|
| | NC | PC | BA+EO | | |
| Villus height, μm | 354.84 | 392.96 | 411.36 | 37.613 | 0.532 |
| Crypt depth, μm | 291.97 | 270.85 | 275.70 | 13.270 | 0.335 |
| Villus:crypta ratio | 1.255 | 1.443 | 1.513 | 0.165 | 0.490 |

[1]NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%). Data are presented as mean (5 piglets/treatment).

[2]SEM: standard error of the mean.

[3]Data are presented as means (5 piglets/treatment).

**Table 3.** Effects of experimental diets on redox parameters in jejunal mucosa of weaned piglets.

| Item[1] | Treatments[2] | | | SEM | P-value[3] |
|---|---|---|---|---|---|
| | NC | PC | BA+EO | | |
| CAT, nmol $min^{-1}$ mg $prt^{-1}$ | 113.39 | 128.20 | 116.45 | 21.477 | 0.785 |
| SOD, U mg $prt^{-1}$ | 933.65[b] | 1970.14[a] | 854.97[b] | 239.600 | 0.047 |
| GST, mmol $min^{-1}$mg $prt^{-1}$ | 114.42 | 126.08 | 101.84 | 16.430 | 0.563 |
| GSH, µg mg $tissue^{-1}$ | 679.69[ab] | 479.93[b] | 889.55[a] | 85.332 | 0.027 |

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

[2]NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%).

[3]Different lowercase letters indicate significant differences between groups according to Tukey's test, $P < 0.050$. Data are presented as means (5 piglets/treatment).

**Table 4.** Effects of experimental diets on relative gene expression of occludin and GLP-2 in jejunal mucosa of weaned piglets.

| Item[1] | Treatments[2] | | | SEM | P-value[3] |
|---|---|---|---|---|---|
| | NC | PC | BA+EO | | |
| Occludin | 1.000 | 0.976 | 1.104 | 0.207 | 0.885 |
| GLP-2 | 1.000 | 1.319 | 1.136 | 0.227 | 0.574 |

[1]GLP-2, glucagon-like peptide-2; SEM, standard error of the mean.

[2]NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%).

[3]Data are presented as means (5 piglets/treatment).

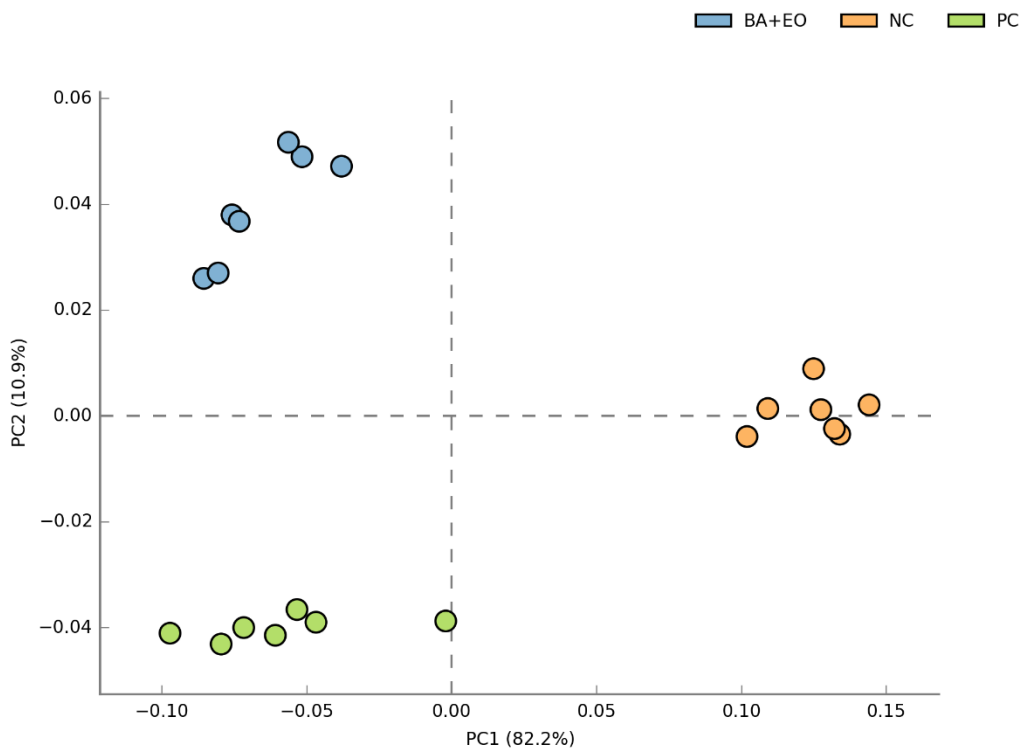


**Figure 4.** Principal components analysis (PCA) of bacterial community structure between NC, PC, and BA+EO groups.

Each symbol represents each gut microbiota. BA+EO: supplementation with benzoic acid and essential oils (0.3%); NC: negative control; PC: positive control (200 ppm colistin sulphate). Piglet is an experimental unit; 7 piglets/treatment.

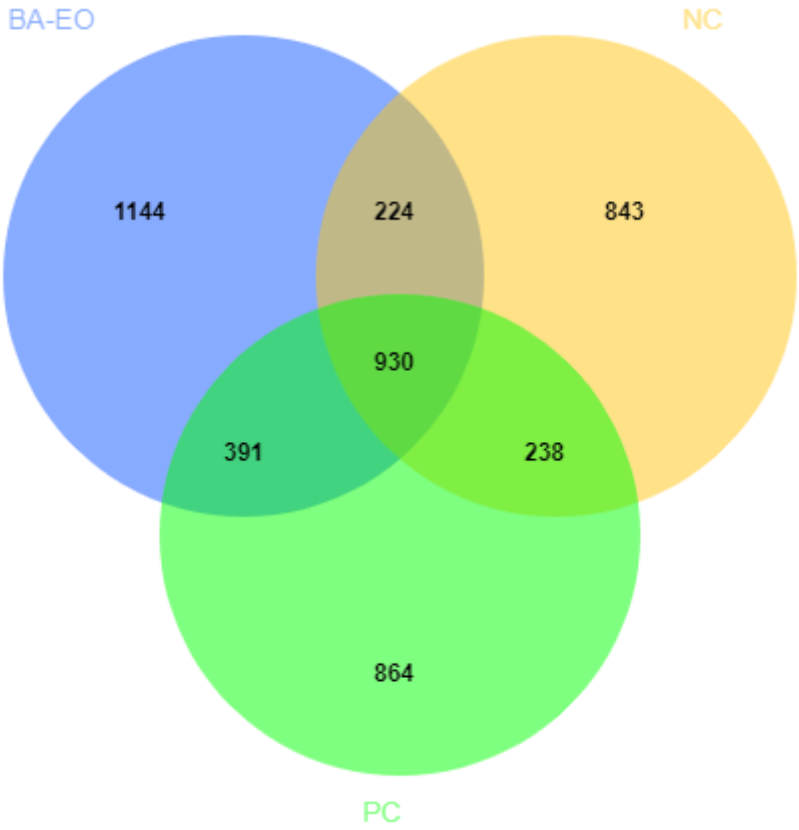


**Figure 5.** Venn diagram depicting biodiversity between treatments.

Values for each treatment refer to the number of OTUs observed ($P < 0.050$). NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%). Piglet is an experimental unit; 7 piglets/treatment.

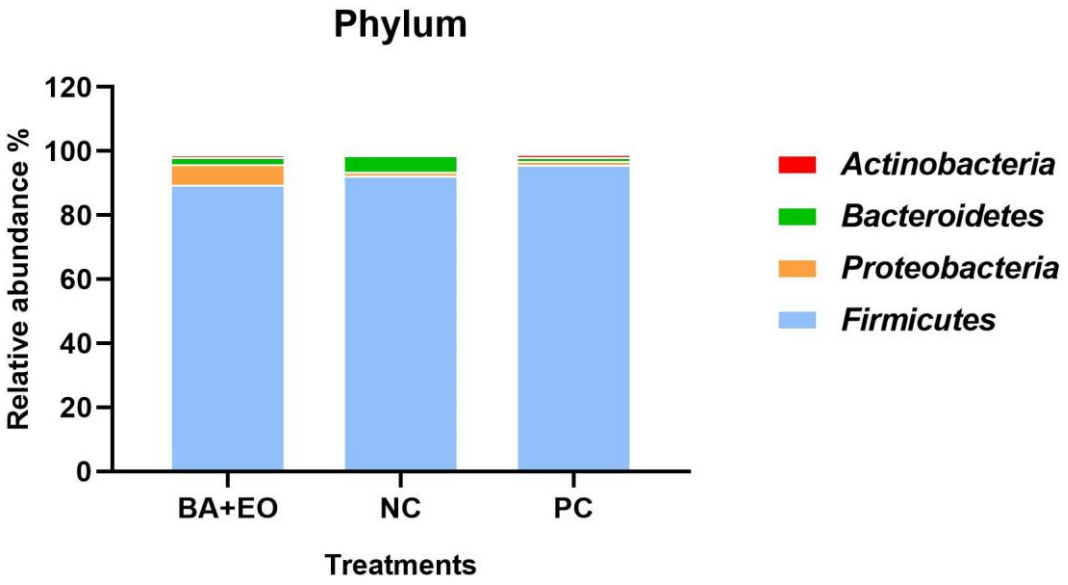


**Figure 6.** Comparison of the phylum levels between treatments.

BA+EO: supplementation with benzoic acid and essential oils (0.3%); NC: negative control; PC: positive control (200 ppm colistin sulphate). The phyla present differ significantly in their abundance between treatments according to the Kruskal Wallis test ($P < 0.050$). Piglet is an experimental unit; 7 piglets/treatment.

**Table 5.** Bacterial genera that varied significantly between different treatments.

| Taxon | p-values* | Treatments[1] | | | | | |
|---|---|---|---|---|---|---|---|
| | | NC | | PC | | BA+EO | |
| | | Mean | SD | Mean | SD | Mean | SD |
| *Mitsuokella* | 0.014 | 0.37 | 0.09 | 0.00 | 0.00 | 0.06 | 0.01 |
| *Christensenella* | 0.019 | 0.13 | 0.04 | 0.84 | 0.14 | 2.37 | 0.24 |
| *Clostridium sensu stricto 1* | 0.019 | 3.83 | 0.41 | 5.06 | 0.35 | 7.88 | 0.68 |
| *Eubacterium eligens* | 0.019 | 0.04 | 0.02 | 0.29 | 0.05 | 0.14 | 0.03 |
| *Megasphaera* | 0.019 | 11.88 | 0.71 | 1.62 | 0.23 | 3.07 | 0.35 |
| *Peptoclostridium* | 0.019 | 0.26 | 0.05 | 0.41 | 0.06 | 0.13 | 0.02 |
| *Prevotella* | 0.019 | 4.91 | 0.41 | 0.25 | 0.05 | 0.92 | 0.11 |
| *Sarcina* | 0.019 | 0.32 | 0.04 | 0.14 | 0.03 | 0.05 | 0.02 |
| *Streptococcus* | 0.019 | 8.82 | 0.51 | 2.30 | 0.18 | 1.20 | 0.07 |
| *Subdoligranulum* | 0.019 | 3.78 | 0.21 | 7.86 | 0.33 | 5.32 | 0.30 |
| *Acetitomaculum* | 0.021 | 0.00 | 0.00 | 0.00 | 0.00 | 0.02 | 0.01 |
| *Candidatus Arthromitus* | 0.021 | 0.00 | 0.00 | 0.00 | 0.00 | 0.05 | 0.02 |
| *Acidaminococcus* | 0.021 | 0.49 | 0.08 | 0.00 | 0.00 | 0.01 | 0.01 |
| *Catenibacterium* | 0.027 | 0.70 | 0.07 | 1.69 | 0.20 | 0.92 | 0.12 |
| *Collinsella* | 0.027 | 0.28 | 0.03 | 0.47 | 0.05 | 0.62 | 0.08 |
| *Clostridium sensu stricto 6* | 0.032 | 0.13 | 0.02 | 0.03 | 0.02 | 0.06 | 0.02 |
| *Ruminococcus 2* | 0.032 | 0.10 | 0.03 | 0.41 | 0.04 | 0.54 | 0.08 |
| *Ruminococcus 1* | 0.033 | 0.47 | 0.06 | 0.95 | 0.14 | 0.65 | 0.07 |
| *Escherichia-Shigella* | 0.037 | 1.12 | 0.12 | 0.88 | 0.11 | 6.64 | 0.46 |
| *Eubacterium nodatum* | 0.037 | 0.01 | 0.01 | 0.13 | 0.01 | 0.03 | 0.01 |
| *Lachnospira* | 0.037 | 0.04 | 0.02 | 0.12 | 0.04 | 0.31 | 0.03 |
| *Treponema 2* | 0.038 | 0.02 | 0.01 | 0.06 | 0.03 | 0.14 | 0.04 |
| *Coprococcus 1* | 0.039 | 0.43 | 0.05 | 0.31 | 0.03 | 0.22 | 0.04 |
| *Fusicatenibacter* | 0.043 | 0.16 | 0.04 | 0.79 | 0.19 | 0.24 | 0.05 |

*Significance value according to the Kruskal Wallis test ($P < 0.050$). Piglet is an experimental unit; 7 piglets/treatment. Data are expressed as means and standard deviations.

[1]NC: negative control; PC: positive control (200 ppm colistin sulphate); BA+EO: supplementation with benzoic acid and essential oils (0.3%).

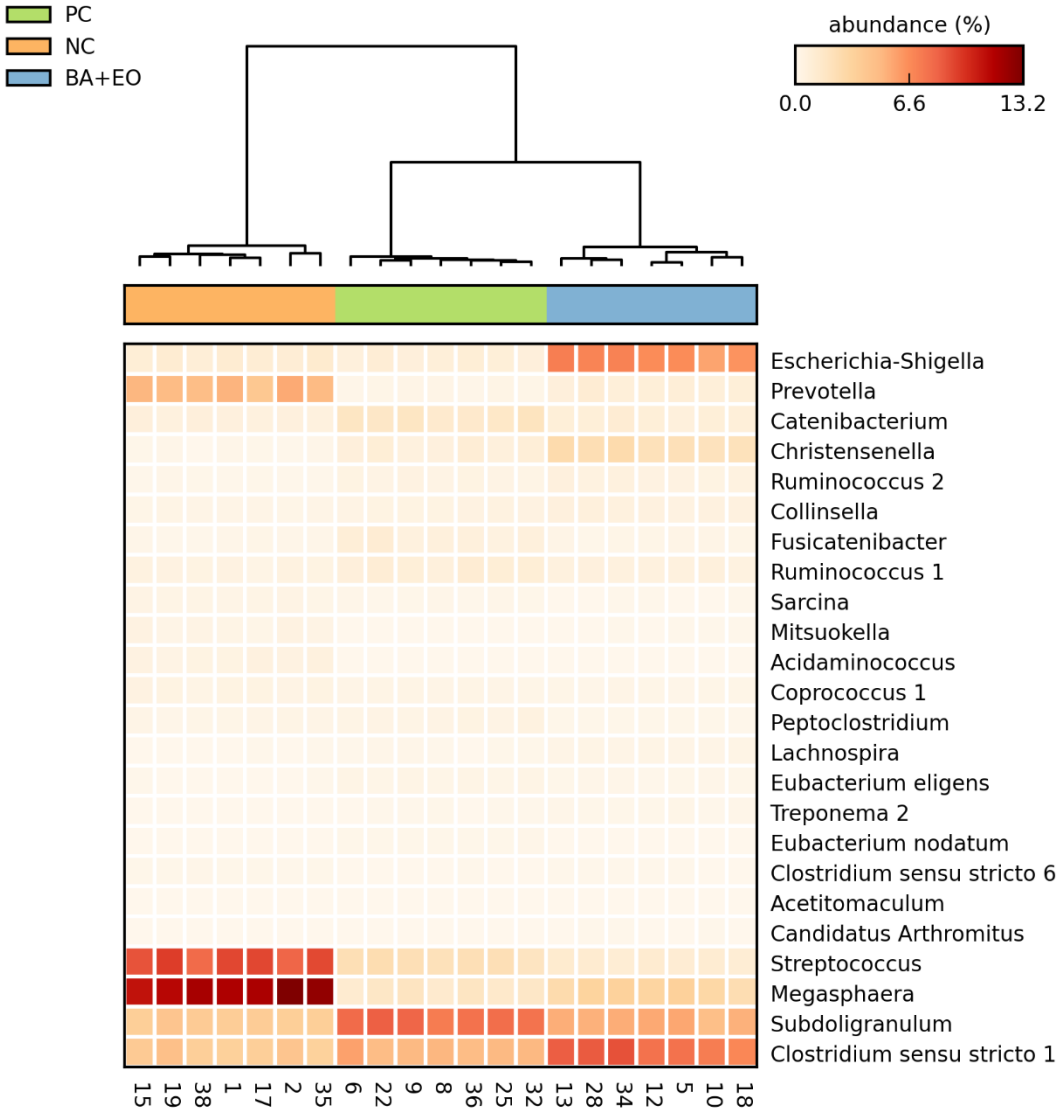


**Figure 7.** Taxa that differ significantly in their abundance between treatments using the Kruskal Wallis test ($P < 0.050$).

PC: positive control (200 ppm colistin sulphate); NC: negative control; BA+EO: supplementation with benzoic acid and essential oils (0.3%). Piglet is an experimental unit; 7 piglets/treatment.

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

| | Nursery | | | | Growth/finish | | | |
|---|---|---|---|---|---|---|---|---|
| | **Phase I** | **Phase II** | **Phase III** | **Phase IV** | **Phase I** | **Phase II** | **Phase III** | **Phase IV** |
| **Ingredients, %** | **(0-7 days)** | **(8-14 days)** | **(15-21 days)** | **(22-42 days)** | **(43-56 days)** | **(57-84 days)** | **(85-107 days)** | **(107-132 days)** |
| Corn, 7.88% CP | 47.214 | 50.436 | 55.000 | 65.000 | 74.334 | 75.292 | 80.984 | 81.987 |
| Soybean meal, 46% CP | 10.000 | 15.000 | 25.000 | 30.000 | 23.500 | 22.000 | 17.000 | 16.000 |
| Plasma spray dried | 5.000 | 2.500 | - | - | - | - | - | - |
| Sugar | 2.500 | 2.000 | 2.500 | - | - | - | - | - |
| Protein whey dried | 15.000 | 12.500 | 5.000 | - | - | - | - | - |
| Enegertic whey dried | 7.895 | 4.303 | 4.500 | - | - | - | - | - |
| Soy protein concentrate | 8.558 | 9.457 | 3.257 | - | - | - | - | - |
| L-Lysine HCl | 0.293 | 0.315 | 0.470 | 0.499 | 0.332 | 0.063 | 0.339 | 0.337 |
| DL-Methionine | 0.183 | 0.187 | 0.217 | 0.201 | 0.071 | 0.500 | 0.055 | 0.052 |
| L-Threonine | 0.067 | 0.085 | 0.169 | 0.179 | 0.114 | 0.315 | 0.122 | 0.119 |
| L-Tryptophan | 0.050 | 0.049 | 0.060 | 0.056 | 0.001 | 0.103 | 0.009 | 0.010 |
| L-Valine | - | - | 0.090 | 0.091 | - | - | - | - |
| Limestone | - | - | - | 1.000 | - | - | - | - |
| Calcium sulfate | 0.550 | - | 0.504 | - | - | - | - | - |
| Dicalcium phosphate | 0.502 | 0.980 | 1.000 | 0.621 | 0.528 | 0.537 | 0.397 | 0.401 |
| Salt | 0.250 | 0.250 | 0.300 | 0.400 | 0.500 | 0.570 | 0.500 | 0.500 |
| Mineral-vitamin premix[1] | 0.250 | 0.250 | 0.250 | 0.250 | 0.200 | 0.200 | 0.200 | 0.200 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ronozyme Proact | 0.020 | 0.020 | 0.020 | 0.020 | - | - | - | - |
| Ronozyme Histarch | 0.013 | 0.013 | 0.013 | 0.007 | - | - | - | - |
| Ronozyme WX | 0.005 | 0.005 | 0.005 | 0.005 | - | - | - | - |
| Ronozyme Hyphós | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 |
| Antioxidant | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 | 0.010 |
| Sweetener | 0.055 | 0.055 | 0.050 | - | - | - | - | - |
| Flavoring | 0.075 | 0.075 | 0.075 | - | - | - | - | - |
| Inert | 1.500 | 1.500 | 1.500 | 1.651 | 0.400 | 0.400 | 0.400 | 0.400 |
| **Calculated values** | | | | | | | | |
| ME, Kcal/kg | 3553.830 | 3448.540 | 3385.300 | 3238.760 | 3233.409 | 3217.592 | 3251.245 | 3253.116 |
| Crude protein, % | 20.463 | 21.302 | 19.947 | 20.118 | 17.221 | 16.567 | 14.712 | 14.319 |
| SID[2] Lys, % | 1.400 | 1.400 | 1.320 | 1.300 | 1.000 | 0.950 | 0.850 | 0.825 |
| SID Met, % | 0.466 | 0.481 | 0.486 | 0.472 | 0.300 | 0.285 | 0.255 | 0.248 |
| SID Met + Cys, % | 0.826 | 0.826 | 0.779 | 0.767 | 0.570 | 0.547 | 0.497 | 0.485 |
| SID Thr, % | 0.910 | 0.910 | 0.858 | 0.845 | 0.650 | 0.618 | 0.570 | 0.553 |
| SID Trp, % | 0.280 | 0.280 | 0.264 | 0.260 | 0.170 | 0.162 | 0.145 | 0.140 |
| Lactose, % | 13.500 | 10.385 | 5.210 | - | - | - | - | - |
| Total calcium, % | 0.625 | 0.625 | 0.625 | 0.749 | 0.316 | 0.526 | 0.271 | 0.270 |
| Available phosphorus, % | 0.483 | 0.549 | 0.494 | 0.380 | 0.330 | 0.330 | 0.300 | 0.300 |

[1]Levels per kg of premix. Minerals: 52 g of iron, 23 g of manganese, 102 mg of cobalt, 57.5 g of zinc, 665 mg of iodine, 184 mg of selenium, 7,500 mg of cooper, 100 mg of chromium. Vitamins: 15,000,000 IU of vitamin A, 3,900,000 IU of vitamin D3, 100,000 IU of vitamin E, 5,000

mg of vitamin K3, 4,000 mg of vitamin B1, 10,000 mg of vitamin B2, 30,000 mg of vitamin pantothenic acid, 6,000 mg of vitamin B6, 50 mg of vitamin B12, 60,000 mg of nicotinic acid, 4,500 mg of folic acid, 500 mg of biotin.

[2]SID = standardized ileal digestible.

**Supplementary table S2.** Primers sequences

| Gene | Accession No | Sequences (5'- 3') | Product length, bp |
|---|---|---|---|
| β-actin | XM_003357928.4 | β F: GCCCGTCCATCGTCCACCG<br>R: CAGGAGGCTGGCATGAGGTGTG | 127 |
| RPL-32 | NM_001001636.1 | F: CAAAATTAAGCGGAACTGGCGG<br>R: GCACATTAGCAGCACTTCAAGC | 187 |
| GLP-2 | NM_214324.1 | F: CCAGGATTTTGTGCAGTGGC<br>R: TCCTCTGGGAAATCTCGCCT | 192 |
| Occludin | NM_001163647.2 | F: GCAGCAGTGGTAACTTGGA<br>R: GTCGTGTAGTCTGTCTCGTAATG | 106 |

# CHAPTER 3

## ARTICLE 2 - A NEW APPROACH: PREVENTIVE PROTOCOLS WITH YEAST PRODUCTS AND ESSENTIAL OILS CAN REDUCE THE IN-FEED USE OF ANTIBIOTICS IN GROWING-FINISHING PIGS

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

**https://www.elsevier.com/journals/livestock-science/1871-1413/guide-for-authors**

## RESUMO

Estratégias nutricionais vêm sendo estudadas na tentativa de minimizar o uso de antibióticos via ração na produção suína. Nossa hipótese é que a substituição de antibióticos promotores de crescimento (APC) por protocolos preventivos com aditivos nutricionais ou a redução na frequência do uso de antibióticos profiláticos, devido a inclusão destes aditivos, não interfere no desempenho dos suínos em crescimento e terminação e modifica a microbiota fecal de suínos em idade de abate. O objetivo deste estudo foi avaliar o uso de produtos à base de levedura (LEV) e óleos essenciais (OE) em substituição total ou parcial aos protocolos de antibióticos via ração (promotores de crescimento e profiláticos), tanto em doses recomendadas quanto em superdose dos antibióticos profiláticos, no desempenho e incidência de diarreia em suínos em crescimento e terminação; e na microbiota fecal de suínos em idade de abate. Quatrocentos suínos (20,36 ± 2,64 kg) foram distribuídos em um delineamento em blocos casualizados (8 repetições): dietas com antibióticos profiláticos e promotor de crescimento (ANT); ANT com 30% a mais de antibióticos profiláticos (ANT+30); dietas com menos profiláticos e LEV (ANT+LEV); dietas com menos profiláticos, LEV e OE (ANT+LEV+OE); dietas sem antibióticos com LEV e OE (LEV+OE). Dos 0 aos 14 dias, os suínos dos grupos ANT+30, ANT+LEV e ANT+LEV+OE apresentaram um maior peso ao final da fase e ganho de peso diário (GPD) do que os suínos do grupo LEV+OE. Aos 35 dias, os suínos dos tratamentos ANT+30 e ANT+LEV+OE apresentaram maior peso do que o grupo LEV+OE. Aos 105 dias, suínos do grupo ANT tiveram um peso maior que os suínos do grupo LEV+OE. Durante o período total, os suínos do grupo ANT apresentaram maior GPD e melhor conversão alimentar (CA) do que o grupo LEV+OE. Dos 0 a 35 dias, os suínos do tratamento LEV+OE apresentaram uma maior incidência de diarreia do que os demais grupos. Dos 49 aos 70 dias, os tratamentos ANT+LEV e ANT+LEV+OE mostraram uma incidência de diarreia menor do que o grupo LEV+OE, o que permaneceu durante todo o período. Aos 105 dias, a α-diversidade da microbiota fecal, feita por Entropia de Shannon, foi menor nos grupos ANT+30 e LEV+OE do que a observada para o grupo ANT+LEV+OE. A abundância do filo *Firmicutes* e a razão *Firmicutes/Bacteroidetes* foram maiores para os suínos do grupo ANT do que o grupo ANT+LEV+OE. A abundância do filo *Proteobacteria* para o tratamento ANT+LEV+OE foi maior do que para os grupos ANT, ANT+LEV e LEV+OE. A abundância relativa da família *Peptostreptococcaceae* foi maior nos grupos ANT, ANT+30 e ANT+LEV do que nos grupos ANT+LEV+OE e LEV+OE e os grupos ANT+LEV+OE e LEV+OE mostram uma abundância menor do gênero *SMB53* do que os grupos ANT e ANT+30. Em conclusão, o uso de LEV e OE (promotor de crescimento e profilático), em substituição parcial aos protocolos de antibióticos via ração, não reduz o desempenho de crescimento, pode substituir os promotores de crescimento de antibióticos e reduzir a uso de antibióticos profiláticos via ração para suínos em crescimento e terminação. O uso de LEV e OE, juntamente com antibióticos profiláticos, aumenta a diversidade microbiana, apesar de apresentar gêneros importantes para ganho de peso em menor abundância. A superdose de antibióticos profiláticos não melhora o desempenho e reduz a diversidade microbiana, o que não o caracteriza como um protocolo preventivo eficiente.


**Palavras-chave:** Leveduras. Óleos essenciais. Suínos. Diarreia. Microbioma.

**A new approach: Preventive protocols with yeast products and essential oils can reduce the in-feed use of antibiotics in growing-finishing pigs**

Maíra Resende[a], Rhuan Filipe Chaves[a], Jéssica Aparecida Barbosa[a], Athos Silveira Marques[a], Thamires Allue Dantas[a], Cesar Augusto Pospissil Garbossa[b], Matheus de Oliveira Costa[c], Fernanda Rigo[d], Eliana Ottati Nogueira Dantas[e], Vinícius de Souza Cantarelli[a*].

[a]Animal Science Department, Federal University of Lavras, Lavras, Brazil

[b]Department of Animal Nutrition and Production, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, Brazil

[c]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

[d]Imunova Análises Biológicas, Curitiba, Brazil

[e]Biorigin, São Paulo, Brazil

[*]Corresponding author

E-mail address: vinicius@ufla.br

**ABSTRACT:** The objective of this study was to evaluate the effects of yeast products (YP) and essential oils (EO) in total or partial replacement to in-feed antibiotic protocols (growth promoter and prophylactic), both in recommended doses and in overdose of prophylactic antibiotics, on growth performance, and diarrhea incidence in the growing-finishing pigs; and fecal microbiota in market hogs. Four hundred pigs (20.36 ± 2.64 kg) were assigned to five treatments in a randomized block design: diets with prophylactic and growth promoter antibiotics (ANT); ANT with 30% more prophylactic antibiotics (ANT+30); diets with less prophylactic antibiotics and YP (ANT+Y); diets with less prophylactic antibiotics, YP and EO (ANT+Y+EO); and antibiotics free diets with YP and EO (Y+EO). From 0 to 14d, pigs of the ANT+30, ANT+Y, and ANT+Y+EO treatments showed a greater body weight (BW) and average daily gain (ADG) compared to pigs from the Y+EO group. From 14 to 35d, pigs of ANT+30 and ANT+Y+EO treatments were heavier than Y+EO group. At 105d, ANT pigs had a higher BW than the Y+EO group. For the entire period, ADG of ANT pigs were greater and feed conversion ratio (FCR) better than Y+EO pigs. From 0 to 35d, pigs of the Y+EO treatment showed a higher diarrhea incidence compared to pigs of the other groups. From 49 to 70d, ANT+Y and ANT+Y+EO treatments showed a lower diarrhea incidence than Y+EO group, which remained the case during the overall period. At 105d, the alpha diversity of fecal microbiota by Shannon Entropy were lower in ANT, ANT+30 and Y+EO groups than observed for ANT+Y+EO group. Abundance of *Firmicutes* phylum and *Firmicutes/Bacteroidetes* ratio were higher in ANT than in ANT+Y+EO pigs. *Proteobacteria* phylum abundance in ANT+Y+EO was higher than ANT, ANT+Y, and Y+EO. *Peptostreptococcaceae* family abundance was higher in ANT, ANT+30, and ANT+Y groups than in ANT+Y+EO and Y+EO groups. ANT+Y+EO and Y+EO groups show a lower abundance of *SMB53* genus than ANT and ANT+30 groups. In conclusion, the use of YP and EO, in partial replacement to the in-feed antibiotic protocols, does not reduce the growth performance, can replace antibiotic growth promotors, and reduce the in-feed use of prophylactic antibiotics in growing-finishing pigs. The use of YP and EO, together with prophylactic antibiotics, increase the microbial diversity, despite having important genera for weight gain in less abundance. Overdose of prophylactic antibiotics does not improve growth performance and reduces microbial diversity, which does not characterize it as an efficient preventive protocol.

**Keywords**: Prebiotics, growth promoters, diarrhea, microbiome, swine.

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## 1. Introduction

In-feed antibiotics have been widely used to increase growth rates and prevent diseases for pigs (Van Boeckel et al., 2015), as much in the post-weaning period as in the growing and finishing phases (Gaskins et al., 2002). However, routine use of antibiotics, and the overdose mainly, in food-producing animals has significantly contributed to the increasing emergence of multi-drug resistant pathogens, incurring a major health concern in both animals and humans (Landers et al., 2012; Tang et al., 2017). There is currently a great interest in the production of pigs without antibiotics (Liu et al., 2018). Therefore, to avoid the negative effects of removing antibiotics from diets for pigs, changes in management and nutritional strategies may be required (Kil and Stein, 2010), as well as the use of validated alternative additives (Heo et al, 2013; Liu et al., 2018).

Yeast products are proposed as alternatives to antibiotics in the livestock industry (Burdick Sanchez et al., 2021). Dietary supplementation of YP has been paid increasing attention for improving immune function and intestinal development in swine (Zhaxi et al., 2020; Xu et al., 2018; Broadway et al., 2015). β-glucan, a functional polysaccharide of D-glucose monomers linked by β-glycosidic bonds (Stier et al., 2014), can modulate the immune system and stimulates a cascade of pathways that enhance both innate and adaptive immune responses (Vannucci et al., 2013), besides promotion on intestinal function (Xiong et al., 2015). Mannan oligosaccharides (MOS) prevent the adhesion of pathogenic bacteria to intestinal epithelial cells by attachment to the mannose-binding proteins expressed on the bacterial fimbriae (Kogan and Kocher, 2007; Spring et al., 2015). In addition, MOS supplementation evidenced additional beneficial properties such as decreased incidence of diarrhea (Song et al., 2019; Valpotić et al., 2016; Zhao et al., 2012) and higher growth performance (Agazzi et al., 2020; Miguel et al., 2004).

Essential oils are promising blends as novel antibacterial agents that can be used in pig production. Their bioactive compounds derived have immune, antioxidative, and

antimicrobial properties (Sharifi-Rad et al. 2017). Essential oils enhance digestibility (Chitprasert and Sutaphanit, 2014, Liu et al., 2012), reduce the production of cells and molecules involved in the immune response (Liu et al., 2013; Brenes and Roura, 2010), and promote gut health by minimizing the effect of the pathogenic bacteria (Zhang et al, 2020; Chitprasert and Sutaphanit, 2014). The modulation of gut microbiota, resulting in improvement of the growth performance, also are observed in piglets supplemented with EO (Li et al., 2018; Omonijo et al., 2018), therefore are so studied as alternatives to antibiotics (Gong et al., 2013; Zhang et al., 2020). Studies that associate YP with EO are not found, as well as protocols with different inclusions of antimicrobial additives. In addition, partial replacement of prophylactic antibiotics with additive protocols has not yet been demonstrated. We understand that there is a potential synergism in this association that can contribute beneficially to the growth performance of pigs and reduce the total use of antibiotics (growth promoter and prophylactic forms).

The involvement of gut microbiota in host metabolism and health is well accepted (Lynch and Pedersen, 2016), but some specific roles remain to be studied. The intestinal microbiota plays crucial functions in nutrient digestion and absorption (Backhed et al., 2015), the development of host immune (Postler and Ghosh, 2017), the differentiation of intestinal epithelium (Sommer and Backhed, 2013), and the maintenance of intestinal mucosal barrier (Garrett et al., 2010). There also are a possible link between the intestinal microbiota and growth performance, mainly feed efficiency (FE) in pigs (Tan et al., 2018; McCormack et al., 2017; Xiao et al., 2017; Yang et al., 2016). Therefore, the association of performance tests with microbiome technology in market hogs treated with different preventive protocols can provide a solid contribution to the understanding of the microbiota and host metabolism relationship.

We hypothesized that replacing antibiotic growth promoters (AGP) with nutritional additives or reducing the frequency of use of prophylactic antibiotics, due to the inclusion of these additives, do not decrease the growth performance of pigs in the growing-finishing phase and modify the fecal microbiota in market hogs. Therefore, the objective of this study was to evaluate the effects of YP and EO in total or partial replacement to the in-feed antibiotic protocols (growth promoter and prophylactic), both in recommended doses and in overdose of prophylactic antibiotics, on growth performance, diarrhea incidence in the growing-finishing pigs, and fecal microbiota in market hogs.

## 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 University of São Paulo under Protocol number 2172090321. The experiment was conducted in the growing-finishing facilities of the Animalnutri Research Center located within a commercial pig farm in Patos de Minas, Brazil. A total of two hundred barrows and two hundred gilts (DanBred sows and LQ1250 sires) with an average initial body weight of 20.36 ± 2.64 kg (63d of age) were randomly allocated in a randomized block design, with five treatments: diets with prophylactic and growth promoter antibiotics (ANT); ANT with 30% more prophylactics antibiotics (ANT+30); diets with less prophylactics antibiotics and YP as prophylactic and growth promoter (ANT+Y); diets with less prophylactics antibiotics, YP and EO as prophylactic and growth promoter (ANT+Y+EO); and diets free antibiotics and with YP and EO as prophylactics and growth promoter (Y+EO). The treatments are further detailed in Table 1. Eight replicates (10 pigs/pen) were used in the trial. The prophylactic antibiotics was composed by 200 ppm tiamulin and 440 ppm amoxicillin, and the overdose was 30% higher prophylactic antibiotics. The AGP used was 10 and 5 ppm enramycin in growing and finishing phases, respectively. The content of the active components of the yeast products 1 (YP1) was 60% purified β-1,3/1,6-glucans extracted from *Saccharomyces cerevisiae* yeast (Macrogard®, Biorigin, São Paulo, Brazil). The content of the active components of the yeast products 2 (YP2) was 20% functional water-soluble MOS (HyperGen®, Biorigin, São Paulo, Brazil). The content of the active components of the yeast products 3 (YP3) was 18% MOS, extracted from *Saccharomyces cerevisiae* yeast (ActiveMOS®, Biorigin, São Paulo, Brazil). The contents of the active components of the EO were the blend of 12% carvacrol and 6% cinnamaldehyde, capsaicin, anethole, and cineole (Activo®, GRASP, Curitiba, Brazil).

The animals were housed in pens allowed a floor space of 1.40m² per pig and had a partially slatted floor. All piglets were provided with feed and water in a five-space feeder (semi-automatic) and nipple drinkers.

### *2.2 Diets and experimental procedures*

The experimental period was of 105d and it was used six diets, all pigs were fed the same basal diet. 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) and the additives were added according to each treatment. The pigs had *ad libitum* access to feed and water throughout the experimental period. The basal diet did not contain growth promoters' additives.

Feed intake per pen and individual body weights were recorded at 0, 14, 35, 49, 70, 84, and 105d. Based on these data, the ADG, ADFI, and FCR were calculated. For fecal scoring, the feces present in the pen were assessed daily 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 the trial, the occurrence of diarrhea was calculated as percentage of the phase.

*2.3 Sample collections*

On day 104 of the trial, fecal samples were collected from the six average weight replicates (pens) at 84 days, totaling 36 samples. The three pigs with the closest individual ADG to the group ADG mean were chosen. Fecal samples were collected by digital rectal stimulation and a pool with these three samples was formed. All fecal samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses.

*2.4 Fecal microbiota analyses*

Total bacterial DNA was extracted from the fecal samples contents by using a commercial kit (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 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 (Degnan and Ochman, 2012). Sequencing was performed using an Illumina MiSeq® sequencer (Illumina) obtaining paired-end reads of 250 bp as described.

For the sequences obtained, the data filtering was completed by removing low quality base. The sequences after trimming were analyzed with the QIIME pipeline (Caporaso et al., 2010), 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 2017 update (SILVA 128) of the SILVA ribosomal sequences database (Yilmaz et al., 2014) was used. To generate the classification of bacterial communities by identifying OTUs, 22,110 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 sample bias.

*2.5 Nucleotide sequence accession number*

All the raw sequences obtained after assembling and filtering were submitted to the NCBI site under accession number BioSample: PRJNA721984.

*2.6 Statistical analyses*

The pen was used as the experimental unit for the statistical analysis of growth performance and the pooled samples were used as the experimental unit for the microbiome analyses. The data were analyzed using the SAS software statistical package 9.3 (SAS, Cary, NC), except for sequencing data. The Shapiro-Wilk test was used to evaluate parametric data distribution. If the data did not have 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 diarrhea incidence, a generalized linear model (binomial analysis) was performed using the GENMOD procedure of SAS 9.3, with a significance level of 0.050. DNA sequencing were performed using the statistical metagenomics program STAMP: Statistical Analysis of Metagenomic Profiles (Parks et al., 2014). To compare the abundance of the genders identified between treatments, ANOVA ($P < 0.050$) with

Tukey-Kramer post-hoc test and Benjamini-Hochberg FDR multiple test correction was used. 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. The *Firmicutes/Bacteroidetes* (F/B) ratio means between different groups was compared by one-way ANOVA ($P < 0.050$) with Tukey's multiple comparisons test. The microbiome figures and their legends were automatically generated by the STAMP program.

## 3. Results

### *3.1 Growth performance*

The growth performance results of the pigs during the experimental period are presented in Table 2. In the growing I phase (0 to 14d), the pigs of the ANT+30, ANT+Y, and ANT+Y+EO treatments showed a greater BW ($P = 0.001$) and ADG ($P = 0.001$) compared to the pigs from the Y+EO group. The pigs of ANT+Y+EO group had better ADG than ANT pigs. In the growing II phase (14 to 35d), the pigs of the ANT+30 and ANT+Y+EO treatments were heavier than Y+EO group ($P = 0.010$). In the last phase (finishing II, 84 to 105d), the ANT pigs had a higher BW than the Y+EO group ($P = 0.023$), but similar results to ANT+30, ANT+Y, and ANT+Y+EO treatments. In this phase, the pigs of ANT+30 and Y+EO groups had lower ADG than ANT pigs ($P = 0.007$). When taking in account the entire experimental period, the ADG of the ANT pigs were greater than Y+EO pigs but similar results to the ANT+30, ANT+Y, and ANT+Y+EO groups ($P = 0.025$). The pigs of ANT group had better FCR than Y+EO pigs and similar to other treatments ($P = 0.011$)

### *3.2 Diarrhea incidence*

As shown in Figure 1, during the growing phase I (0 to 14d) and II (14 to 35d), the pigs of the Y+EO treatment showed a higher diarrhea incidence compared to the pigs of the other groups ($P < 0.001$). In growing phase III (35 to 49d), the ANT, ANT+30 and ANT+Y+EO treatments had a lower diarrhea incidence than the pigs of Y+EO group ($P = 0.019$). From 49 to 70d (growing phase IV), the ANT+Y and ANT+Y+EO treatments showed a lower diarrhea incidence than the Y+EO group ($P = 0.005$), which remained the case during the overall period ($P < 0.001$). Considering the

entire period, the ANT+Y+EO group had a lower diarrhea incidence than the ANT+Y group. The treatments had no effects on the diarrhea incidence from the 70$^{th}$ to 105$^{th}$ day of the trial ($P = 0.631$).

*3.3 Fecal microbiota*

Regarding the biodiversity indicators of the bacterial communities, alpha diversity by Shannon Entropy were lower in the ANT, ANT+30, and Y+EO groups than observed for the ANT+Y+EO group ($P < 0.050$), but similar between other groups (Figure 2). The most abundant phyla on fecal samples were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria,* and *Spirochaetes* (Figure 3). *Firmicutes* phylum and F/B ratio were higher abundant in the ANT group than in the ANT+Y+EO group ($P < 0.050$, Figure 4). *Proteobacteria* phylum relative abundance in ANT+Y+EO was similar to ANT+30 and higher than ANT, ANT+Y, and Y+EO ($P < 0.050$, Figure 5). At lower taxonomic levels representing *Firmicutes* phyla, *Peptostreptococcaceae* family (a member of *Clostridia* Class) relative abundance was higher in ANT+30, ANT, and ANT+Y groups than in the ANT+Y+EO and Y+EO groups ($P < 0.050$, Figure 6). ANT+Y+EO and Y+EO groups show a lower abundance (%) of *SMB53 g*enus (*Clostridiaceae* family) than ANT and ANT+30 groups. Y+EO had shown the lowest levels between all groups ($P < 0.010$; Figure 7). *Alphaproteobacteria* class abundance was higher in the ANT+30 group than any other group ($P < 0.050$. Figure 8).

**4. Discussion**

In-feed additives are used as long as they fulfill a variety of purposes. It is important that they are resistant to the processes used in-feed manufacturing. In addition, they must be safe for animals and must not affect food safety. Those products should also share a positive image towards the public, who are increasingly sensitive to the way livestock production works (Gallois et al., 2009). Finally, these alternatives must be effective in their purpose, act as growth-promoters and provide health benefits to pig. Although, it is important to note that there is no single alternative to substitute in-feed antibiotics, and a combination of different alternatives to antibiotics may be the most promising method to reduce or replace antibiotics in animal feeds (Xu et al., 2021). In this scenario, our objective was to evaluate the reduction, or even the replacement of in-feed antibiotics by associated additives, not only the use as AGP, but

also prophylactic forms. After all, this is already a reality in most of the pig productions around the world.

In this study, in the first two phases of evaluation, the replacement of antibiotics by additives maintained the BW and ADG of the pigs. In addition, the use of overdose promoted a greater BW than the use of additives alone. In the entire period, the total substitution of antibiotics by additives decreased the growth performance of pigs, but the reduction in prophylactic antibiotics use as well as the replacement of AGP by YP and EO additives did not interfere in the growth performance of pigs. In the nursery phase, it is common to observe the replacement of antibiotics by additives without impairing growth performance (Resende et al., 2020; Kiarie et al., 2018; Soler et al., 2018; Xiong et al., 2015). This is mainly due to the improvement in intestinal morphology or digestibility (Long et al., 2018). Modulation of the microbiota is also widely observed (Resende et al., 2020). The changes are associated with the great challenges that weaning provides, as it is associated with intestinal inflammation and digestive disorders (Moeser et al., 2017; Campbell et al., 2013; Smith et al., 2010). In the growing-finishing phase less results are found and can be inconsistent (Wierup, 2001). Cheng et al. (2018) confirm the hypothesis that the use of OE as an alternative to antibiotics can improve intestinal epithelial absorptive function, gut microbiota composition, and antioxidative stress capacity in growing-finishing pigs, as well as Yan et al (2011) when using an herb extract mixture. Xu et al (2021) showed, through a meta-analysis, that plant extracts improve ADG and FCR for growing pigs and can be used as antibiotics substitute. The antimicrobial and anti-inflammatory properties of these additives change the microbiota and the regulates intestinal permeability contributing to their antidiarrheal properties (Wang et al., 2017). We observed a lower diarrhea incidence when EO were used in the protocol associated with antibiotics and YP, which contributes to these findings. However, some studies that evaluate the inclusion of additives, such as EO, plant extracts or YP, in diets free antibiotics, have not observed an effect on the growth performance of pigs (Lowell et al., 2018; Yan et al., 2010; Janz et al., 2007; Davis et al., 2002). This inconsistency may be attributed to the different additives levels and animals used in each study (Yan et al., 2012) or differences of optimum concentration, purity, molecular weight, conformation, chemical modification, and solubility of YP in the diet formulation (Luo et al., 2019). Moreover, in this phase the pigs may have the more developed digestive system, improved

immunity, and increased resistance to intestinal disorders as pig become older (Yan et al., 2010).

The pigs from ANT and ANT+30 groups did not show differences in growth performance in any of the phases, which shows that overdose antibiotics can often be ineffective. Resistance to antibiotics is an inherent side effect associated with the overuse, abuse, or substantial use of ANT (Albernaz-Gonçalves et al., 2021; Manyi-Loh et al., 2018; Williams-Nguyen et al., 2016). When resistant strains are extracted from their host, they are disseminated into the environment, and increase the spread of resistant genes among bacterial populations (Lin et al., 2015). The diverse range of novel antibiotics resistance genes could be accessible to clinically relevant bacteria and play a critical role in the emergence of antibiotics resistance among pathogens (Pehrsson et al., 2013). Besides being ineffective, overdose antibiotics negatively affects the alpha diversity, reducing the total genus content, compared with the replacement of AGP and reduction of antibiotics. Elimination of antibiotics in diet shows a similar effect. Reduced alpha diversity is related to a less mature and stable microbiota, with reduction of functional redundancy (Chen et al., 2017; Trevisi et al., 2021). The loss microbial diversity is referred to as gut dysbiosis (Wilkins et al., 2019) and the use of antibiotics is associated with intestinal microbiota imbalance (Jo et al., 2021; Stecher et al., 2013) and reduced diversity (Knecht et al., 2014). Just like there are still persistent long-term impacts on the intestinal microbiota that remain post-treatment (Yin et al., 2015; Cecilia et al., 2007), our studies showed that antibiotics overuse also strongly impacts the diversity.

The supply of the YP, for pigs that received antibiotics during the growing-finishing phase, did not modify the alpha diversity indicators, differently of the use of YP and EO. Changes in alpha diversity were also not observed by Xu et al (2018), when supplementing weaned piglets with yeast probiotics. The effect of YP on the immune system has been investigated in many studies (Broadway et al., 2015), but the results in the enteric microbiota can be contradictory. In addition, an overall increasing trend in alpha community diversity of the gut microbiome is observed during aging (Wang et al., 2019), suggesting that the swine gut microbiome matures after the finishing phase, and this can contribute to the equivalence between the treatments that included the YP or not. Zhang et al. (2020) and Li et al. (2018) also found no differences in alpha diversity in their studies with OE for weaned piglets. As the increase in microbial diversity in this

study was found with the addition of both additives, we can observe a synergism between them, favoring the intestinal health of these pigs. Microbiome diversity is used as a good indicator of a gut healthy in humans (Menni et al., 2017) and animals (Hildebrand et al., 2013).

The most abundant phylum on fecal samples was *Firmicutes*, and *Firmicutes* phylum and F/B ratio were higher abundant in ANT group than in ANT+Y+EO group. It is usual that F/B ratio in swine gut microbiota gradually increases over time (Kim et al., 2015; Kim et al., 2012). Thus, ANT-treated pigs showed an accelerated increase in the F/B ratio (Kim et al., 2016). Pigs from ANT group received antibiotics the entire experimental period, unlike animals from the ANT+Y+EO group. Lower abundance of *Firmicutes* levels reflects on lower F/B ratios indicating a microbiome that favors gram-negative bacteria such as *Bacteroides*, *Alistipes*, *Parabacteroides*, and *Prevotella* (Stojanov et al., 2020).

*Peptostreptococcaceae f*amily (*Firmicutes* phylum) relative abundance was also higher when the antibiotics was used. The abundance of species of four families of the *Firmicutes* phylum (*Streptococcaceae, Peptococcaceae, Peptostreptococcaceae*, and *Clostridiaceae*) correlated positively with host weight gain (Kim et al., 2016). Firmicutes species metabolize available energy sources more effectively than *Bacteroidetes* species, and consequently promote weight gain (Kallus and Brandt, 2012). Thus, manipulating gut microbial communities could control fat storage in pigs (Guo et al., 2008) and affect growth traits in pigs through host–microbe interactions (Oh et al, 2020; Lu et al., 2018).

ANT+Y+EO and Y+EO groups showed a lower abundance of *Clostridium*-related *SMB53* genera than ANT and ANT+30 groups. Pigs from Y+EO group, which did not receive antibiotics at any phase, had shown the lowest levels between all groups. Gao et al (2018) found functional analysis during the weaning and in nursery periods that showed a convergent presence of carbohydrate metabolism, amino acid metabolism, DNA replication and repair, and membrane transportation and the genera. The *SMB53* genus belongs to the *Clostridiaceae* family. Most members of this family have the ability to consume mucus- and plant-derived saccharides, such as glucose (Wuest et al., 2011). These findings are interesting because the pigs from Y+EO group showed lower growth performance during the evaluation, which may have happened due to poor use of feed ingredients. The fecal microbiota in high-FE pigs have a greater

capacity to degrade dietary cellulose, polysaccharide, and protein and may have a greater abundance of microbes to promote intestinal health (Quan et al., 2019). In this study, the use of ANT seems to favor the microbiota for this purpose.

Greater FE increases profitability while reducing the environmental impact of pig production (Quan et al., 2020). This is especially important given that pig is one of the major sources of animal protein in human diet. The reduction in the use of antibiotics proposed in our study, and its effects on growth performance, fulfilled these assumptions and becomes a viable option. Furthermore, total withdrawal can be assessed as a possible protocol when requested, without causing major losses in productivity. In recent years, analyzing the pig microbiota has gained interest because it allows for the prediction of the functional and metabolic capacity of such communities, which are believed to impact all aspects of host physiology including nutrient processing, energy harvesting, and growth performance (Quan et al., 2019, Tan et al., 2017). Thus, a better understanding of how the additives interfere in the finishing pig's microbiota is fundamental for the studies in the future since preventive protocols without antibiotics will be more and more demanded.

## 5. Conclusions

In conclusion, our results indicate that the use of yeast products and essential oils (growth promoter and as prophylactic, in partial replacement to the in-feed antibiotic protocols, does not reduce the growth performance, can replace antibiotic growth promotors, and reduce the in-feed use of prophylactic antibiotics in growing-finishing pigs. In addition, the use of yeast products and essential oils increased the microbial diversity, despite having important genera for weight gain in less abundance. Overdose of prophylactics antibiotics does not improve growth performance and reduces microbial diversity, which does not characterize it as an efficient preventive protocol.

## Conflict of interest

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

## CRediT authorship contribution statement

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

Formal analysis, Writing – review & editing. Jéssica Aparecida Barbosa: Investigation, Writing - review & editing. Athos Silveira Marques: Investigation. Thamires Allue Dantas: Investigation. Cesar Augusto Pospissil Garbossa: Writing - review & editing. Matheus de Oliveira Costa: Formal analyses, Writing - review & editing. Fernanda Rigo: Formal analyses, Writing - review & editing. Eliana Ottati Nogueira Dantas: 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.** Experimental treatments.

| Treatments | Growing phase | | | | Finishing phase | |
|---|---|---|---|---|---|---|
| | 0-14 days | 14-35 days | 35-49 days | 49-70 days | 70-84 days | 84-105 days |
| ANT | Prophylactic antibiotics (PA)* | Antibiotics growth promoter (AGP)** | PA | AGP | PA | AGP |
| ANT+30 | Overdose PA*** | AGP | Overdose PA | AGP | Overdose PA | AGP |
| ANT+Y | PA + YP1∞ | YP2£ | YP1 + YP2 | YP3€ | PA + YP1 | YP3 |
| ANT+Y+EO | PA + YP1 | YP2 + 200 ppm EO | YP1 + YP2 + 400 ppm EO | YP3 | PA + YP1 | YP3 + 200 ppm EO |
| Y+EO | YP1 + YP2 + 400 ppm EO¥ | YP2 + 200 ppm EO | YP1 + YP2 + 400 ppm EO | YP3 | YP1 + YP2 + 400 ppm EO | YP3 + 200 ppm EO |

*Prophylactic antibiotics (PA): 200 ppm tiamulin and 440 ppm amoxicillin. **Antibiotics growth promoter (AGP): 10 and 5 ppm enramycin in growing and finishing phases, respectively. ***30% higher prophylactic antibiotic. ∞YP1: 300 ppm of 60% purified β-1,3/1,6-glucans, from *Saccharomyces cerevisiae* yeast. £YP2: 1000 ppm of 20% functional water-soluble MOS. €YP3: 1500 ppm of 18% MOS, from *Saccharomyces cerevisiae* yeast. ¥Blend of essential oils included 12% carvacrol and 6% cinnamaldehyde, capsaicin, anethole and cineole.

**Table 2.** Effects of experimental diets on growth performance in pigs from growing to finishing phase.

| Item[1] | Treatments[2] | | | | | SEM | P-value[3] |
|---|---|---|---|---|---|---|---|
| | ANT | ANT+30 | ANT+Y | ANT+Y+EO | Y+EO | | |
| Initial BW, kg | 20.369 | 20.364 | 20.364 | 20.363 | 20.364 | 1.022 | 0.999 |
| Growing I, d 0 to 14 | | | | | | | |
| BW 14d, kg | 33.237$^{ab}$ | 33.725$^{a}$ | 33.711$^{a}$ | 33.925$^{a}$ | 32.850$^{b}$ | 1.237 | 0.001 |
| ADFI, kg | 1.338$^{ab}$ | 1.390$^{a}$ | 1.393$^{a}$ | 1.402$^{a}$ | 1.323$^{b}$ | 0.052 | 0.092 |
| ADG, kg | 0.919$^{bc}$ | 0.954$^{ab}$ | 0.953$^{ab}$ | 0.968$^{a}$ | 0.891$^{c}$ | 0.020 | 0.001 |
| FCR | 1.458 | 1.456 | 1.456 | 1.445 | 1.484 | 0.035 | 0.672 |
| Growing II, d 14 to 35 | | | | | | | |
| BW 35d, kg | 52.125$^{ab}$ | 53.050$^{a}$ | 52.675$^{ab}$ | 53.381$^{a}$ | 50.409$^{b}$ | 1.681 | 0.010 |
| ADFI, kg | 1.825 | 1.861 | 1.863 | 1.891 | 1.789 | 0.078 | 0.149 |
| ADG, kg | 0.900 | 0.920 | 0.903 | 0.926 | 0.868 | 0.028 | 0.266 |
| FCR | 2.028 | 2.020 | 2.062 | 2.040 | 2.017 | 0.032 | 0.469 |
| Growing III, d 35 to 49 | | | | | | | |
| BW 49d, kg | 67.813 | 68.925 | 67.613 | 68.581 | 66.211 | 1.985 | 0.143 |
| ADFI, kg | 2.494 | 2.535 | 2.426 | 2.450 | 2.361 | 0.151 | 0.248 |
| ADG, kg | 1.121 | 1.134 | 1.067 | 1.125 | 1.088 | 0.045 | 0.577 |
| FCR | 2.222 | 2.245 | 2.273 | 2.267 | 2.183 | 0.066 | 0.750 |
| Growing IV, d 49 to 70 | | | | | | | |
| BW 70d, kg | 91.520 | 91.441 | 90.648 | 91.472 | 89.692 | 2.405 | 0.446 |
| ADFI, kg | 3.109 | 3.051 | 3.043 | 3.068 | 3.020 | 0.226 | 0.691 |
| ADG, kg | 1.129 | 1.077 | 1.062 | 1.074 | 1.085 | 0.038 | 0.126 |
| FCR | 2.750 | 2.892 | 2.863 | 2.818 | 2.779 | 0.105 | 0.198 |
| Finishing I, d 70 to 84 | | | | | | | |
| BW 84d, kg | 108.840 | 108.580 | 108.120 | 108.090 | 106.570 | 2.586 | 0.410 |
| ADFI, kg | 3.477 | 3.398 | 3.574 | 3.501 | 3.526 | 0.184 | 0.189 |
| ADG, kg | 1.218 | 1.224 | 1.234 | 1.187 | 1.205 | 0.024 | 0.680 |
| FCR | 2.813 | 2.784 | 2.905 | 2.950 | 2.929 | 0.118 | 0.127 |
| Finishing II, d 84 to 105 | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BW 49d, kg | 131.430[a] | 129.120[ab] | 129.410[ab] | 129.660[ab] | 127.090[b] | 2.485 | 0.023 |
| ADFI, kg | 3.504[a] | 3.337[b] | 3.372[ab] | 3.423[ab] | 3.427[ab] | 0.126 | 0.048 |
| ADG, kg | 1.129[a] | 1.026[b] | 1.064[ab] | 1.078[ab] | 1.025[b] | 0.020 | 0.007 |
| FCR | 3.108 | 3.221 | 3.177 | 3.181 | 3.311 | 0.133 | 0.109 |
| Total phase, d 0 to 105 | | | | | | | |
| ADFI, kg | 2.654 | 2.614 | 2.629 | 2.650 | 2.601 | 0.133 | 0.472 |
| ADG, kg | 1.060[a] | 1.042[ab] | 1.039[ab] | 1.047[ab] | 1.021[b] | 0.017 | 0.025 |
| FCR | 2.482[b] | 2.505[ab] | 2.528[ab] | 2.527[ab] | 2.563[a] | 0.083 | 0.011 |

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

[2]ANT: basal diet with antibiotics; ANT+30: basal diet with antibiotics in overdose; ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; Y+EO: basal diet with yeast products and essential oils.

[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).

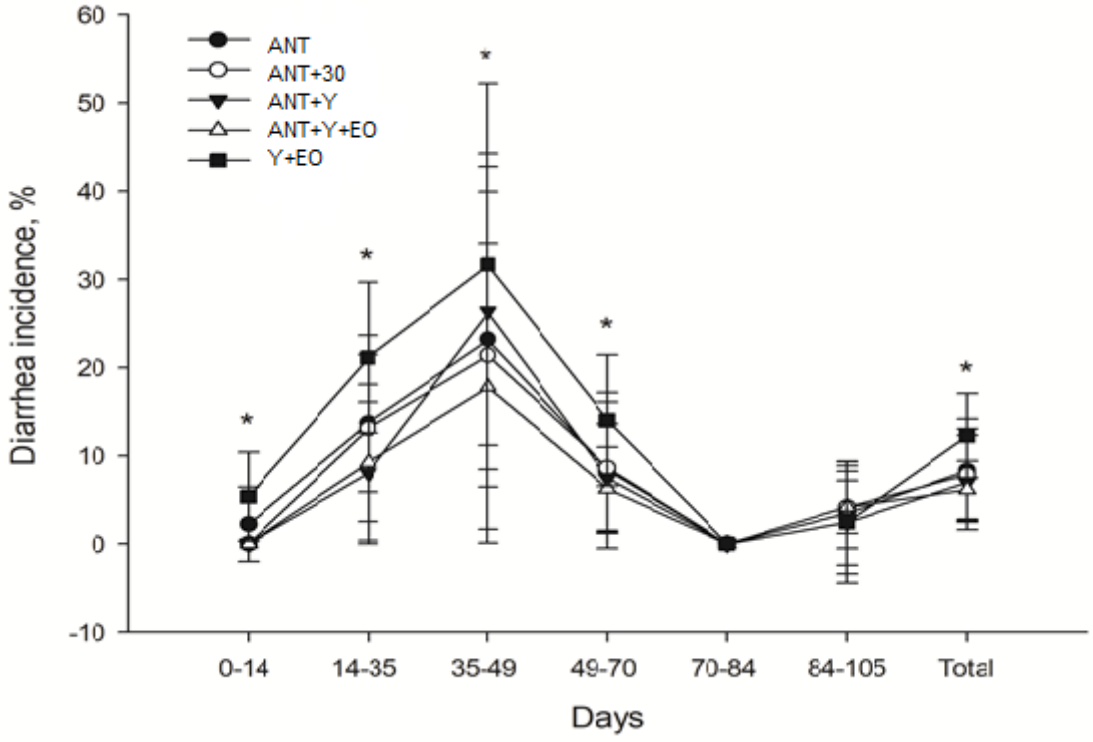


**Figure 1.** Effect of experimental diets on the diarrhea incidence in pigs from growing to finishing phase. ANT: basal diet with antibiotics; ANT+30: basal diet with antibiotics in overdose; ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; Y+EO: basal diet with yeast products and essential oils. Data are expressed as means (8 replicates/treatment) and SEM represented by vertical bars. *Significant differences between groups according to binomial analysis, $P < 0.050$.

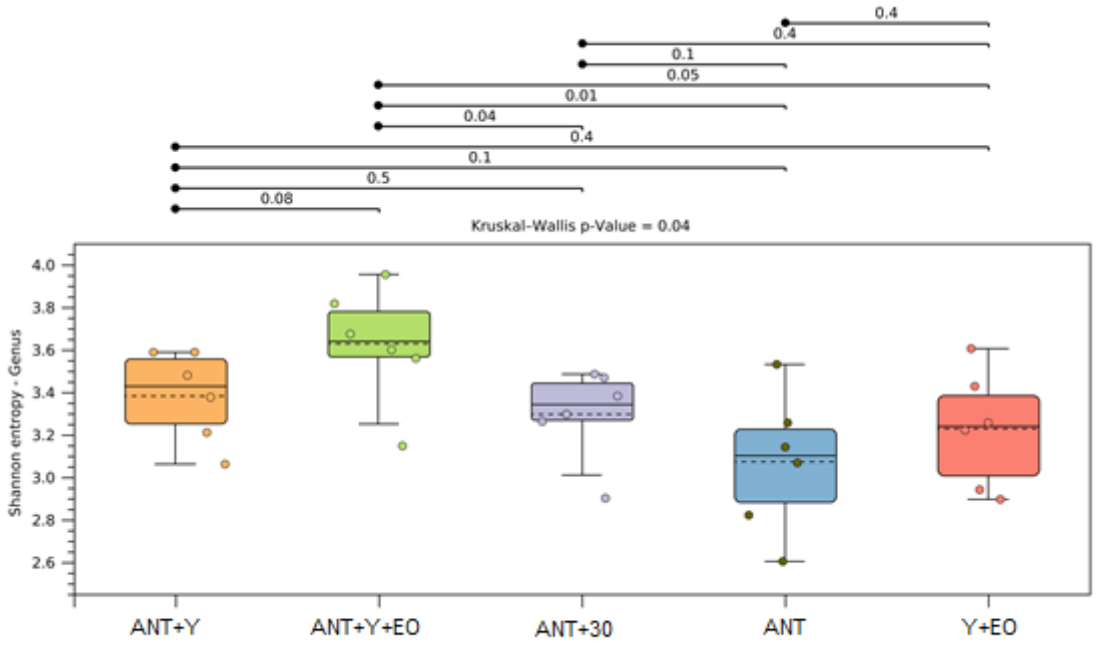


**Figure 2.** Effect of experimental diets on the alpha diversity by Shannon Entropy in market hogs. ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT: basal diet with antibiotics; Y+EO: basal diet with yeast products and essential oils. Piglet is an experimental unit; 6 piglets/treatment.

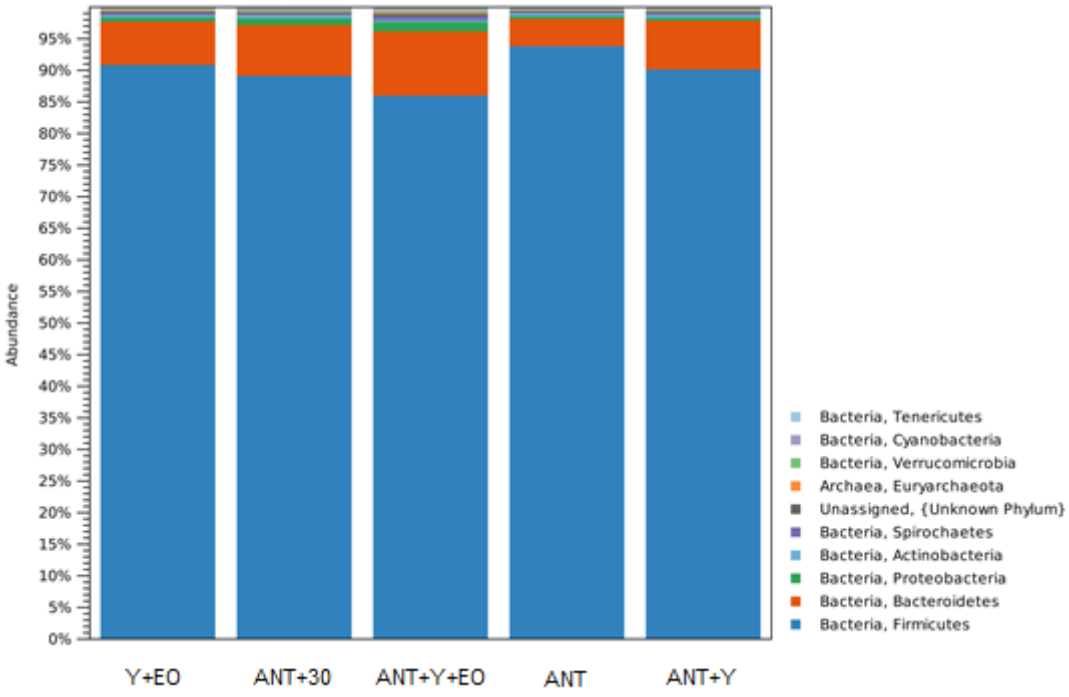


**Figure 3.** Effect of experimental diets on the phylum levels in market hogs. Y+EO: basal diet with yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT: basal diet with antibiotics; ANT+Y: basal diet with antibiotics and yeast products. The phyla present differ significantly in their abundance between treatments according to the Kruskal Wallis test ($P < 0.050$). Piglet is an experimental unit; 6 piglets/treatment.

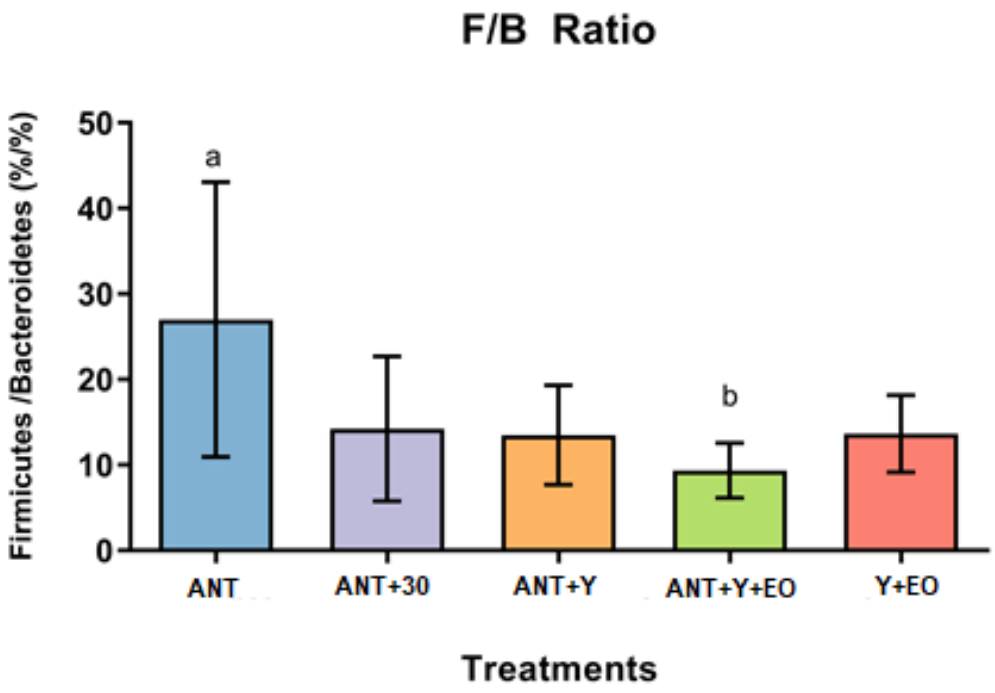


**Figure 4.** Effect of experimental diets on the *Firmicutes/Bacteroidetes* (F/B) ratio in market hogs. ANT: basal diet with antibiotics; ANT+30: basal diet with antibiotics in overdose; ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; Y+EO: basal diet with yeast products and essential oils. The F/B ratio means between different groups was compared by one-way ANOVA ($P < 0.050$) with Tukey's multiple comparisons test. Piglet is an experimental unit; 6 piglets/treatment.

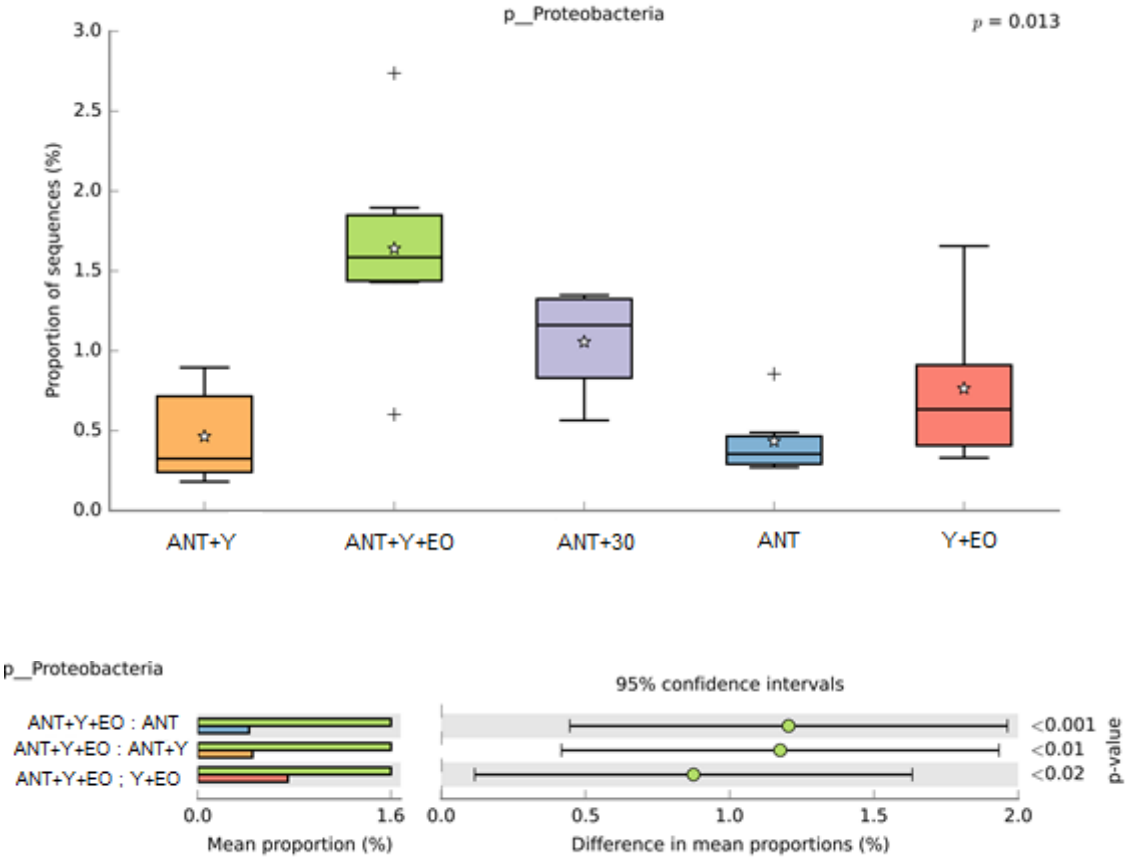


**Figure 5.** Effect of experimental diets on *Proteobacteria* phylum relative abundance in market hogs. (A) Box plot showing the distribution in the proportion of *Proteobacteria* assigned to samples from treatments. (B) Post-hoc plot for *Proteobacteria* indicating. ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT: basal diet with antibiotics; Y+EO: basal diet with yeast products and essential oils. Piglet is an experimental unit; 6 piglets/treatment.

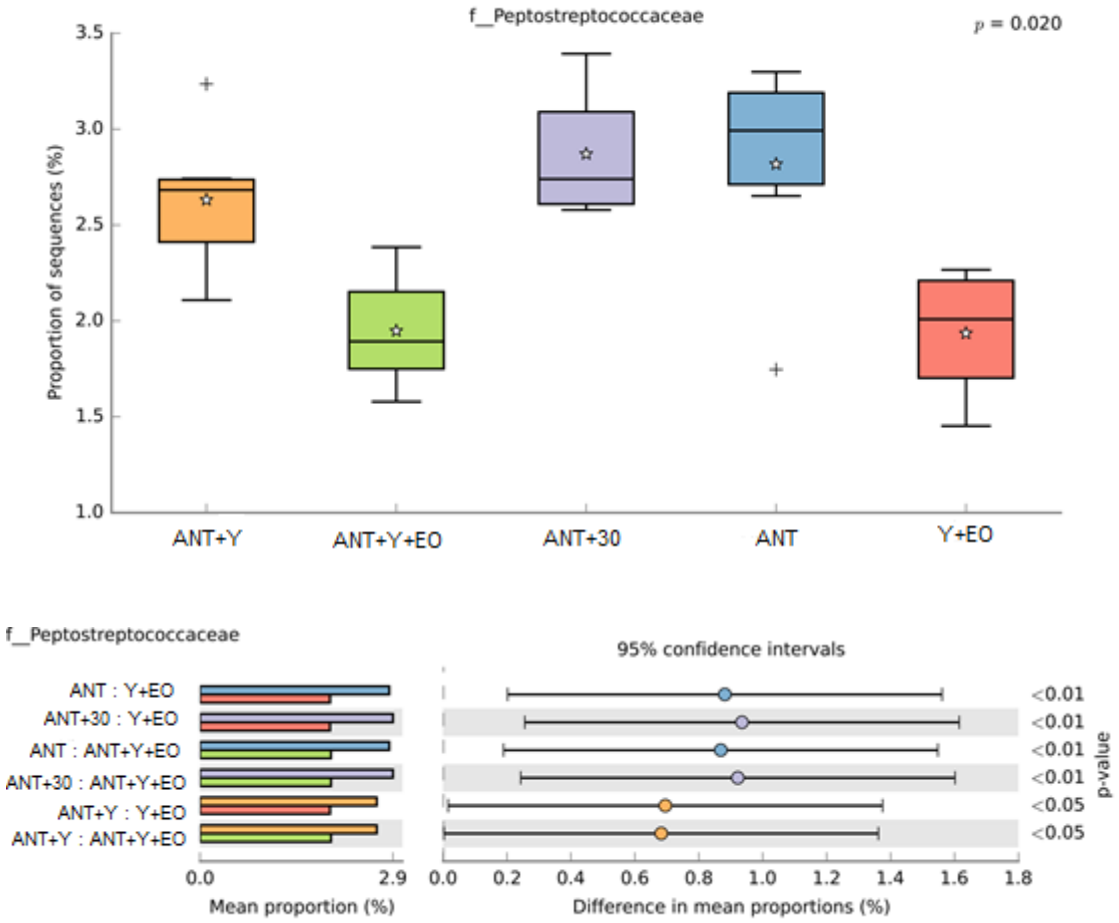


**Figure 6.** Effect of experimental diets on *Peptostreptococcaceae* family relative abundance in market hogs. (A) Box plot showing the distribution in the proportion of *Peptostreptococcaceae* assigned to samples from treatments. (B) Post-hoc plot for *Peptostreptococcaceae* indicating. ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT: basal diet with antibiotics; Y+EO: basal diet with yeast products and essential oils. Piglet is an experimental unit; 6 piglets/treatment.

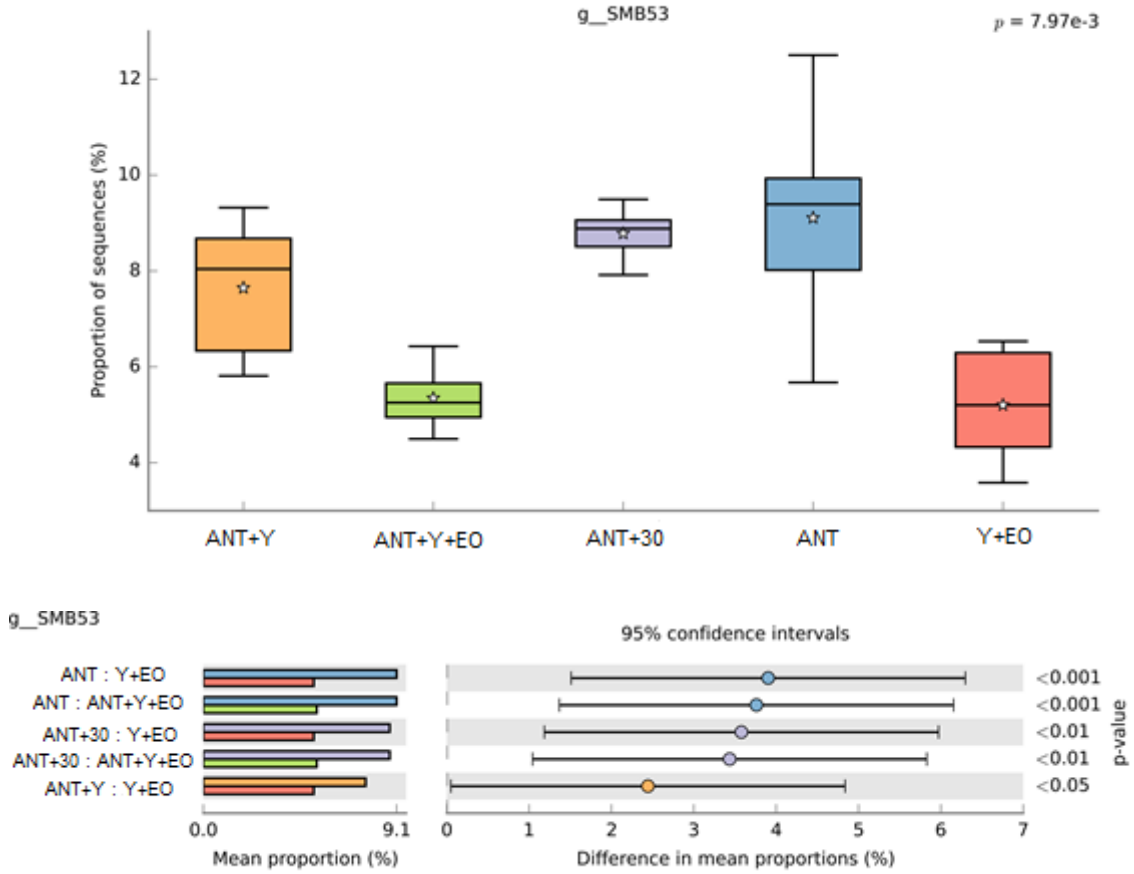


**Figure 7.** Effect of experimental diets on *SMB53* genus (*Clostridiaceae* family) relative abundance in market hogs. (A) Box plot showing the distribution in the proportion of *SMB53* genus assigned to samples from treatments. (B) Post-hoc plot for *SMB53* genus indicating. ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT: basal diet with antibiotics; Y+EO: basal diet with yeast products and essential oils. Piglet is an experimental unit; 6 piglets/treatment.

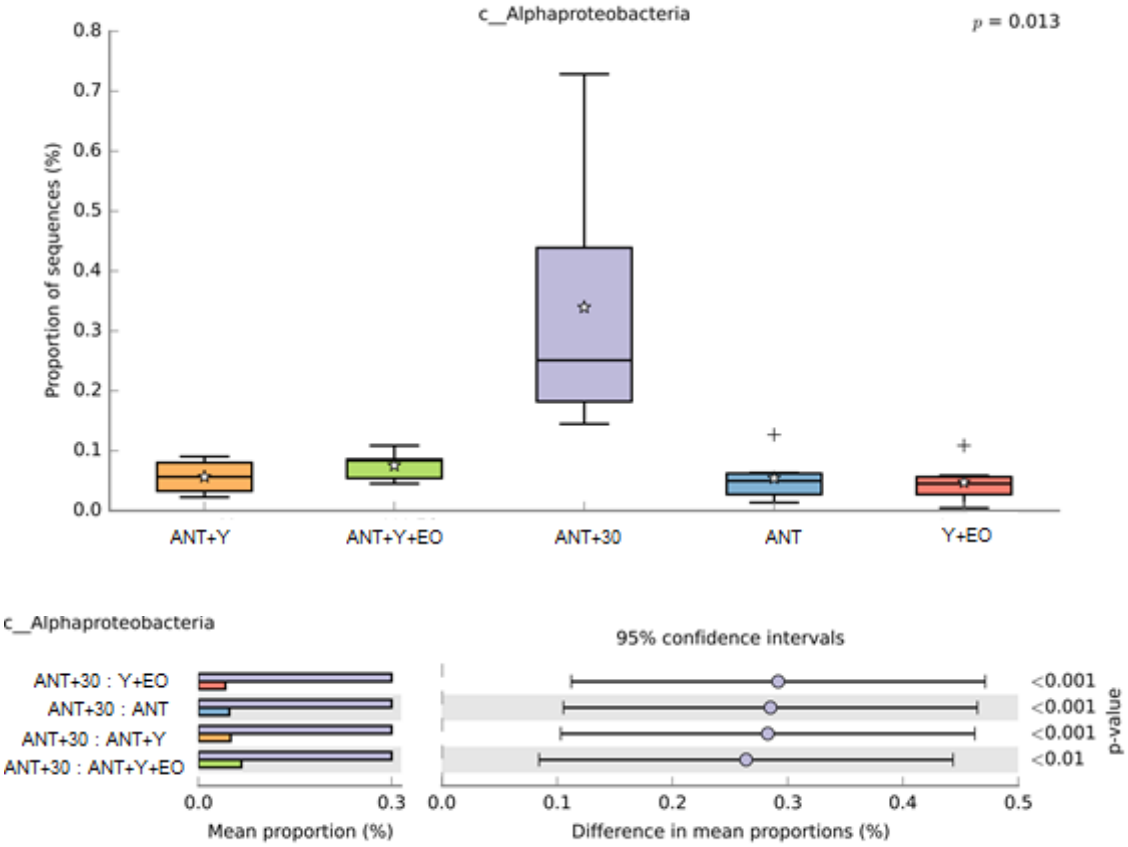


**Figure 8.** Effect of experimental diets on *Alphaproteobacteria* class relative abundance in market hogs. (A) Box plot showing the distribution in the proportion of *Alphaproteobacteria* genus assigned to samples from treatments. (B) Post-hoc plot for *Alphaproteobacteria* genus indicating. ANT+Y: basal diet with antibiotics and yeast products; ANT+Y+EO: basal diet with antibiotics, yeast products and essential oils; ANT+30: basal diet with antibiotics in overdose; ANT: basal diet with antibiotics; Y+EO: basal diet with yeast products and essential oils. Piglet is an experimental unit; 6 piglets/treatment.

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

| | Growing phase | | | | Finishing phase | |
|---|---|---|---|---|---|---|
| | **Phase I** | **Phase II** | **Phase III** | **Phase IV** | **Phase I** | **Phase II** |
| **Ingredients, %** | **(0-14 days)** | **(14-35 days)** | **(35-49 days)** | **(49-70 days)** | **(70-84 days)** | **(84-105 days)** |
| Corn, 7.86% CP[1] | 58.71 | 67.41 | 72.89 | 78.23 | 77.80 | 75.79 |
| Soybean meal, 46% CP | 33.70 | 26.60 | 21.40 | 16.90 | 17.00 | 19.80 |
| Soybean oil | 3.30 | 2.50 | 2.30 | 1.90 | 2.00 | 2.00 |
| Dicalcium phosphate 18.5% P[2] | 1.80 | 1.45 | 1.25 | 1.10 | 1.10 | 1.00 |
| Limestone | 0.76 | 0.66 | 0.60 | 0.56 | 0.56 | 0.52 |
| Salt | 0.47 | 0.43 | 0.40 | 0.40 | 0.40 | 0.35 |
| L-Lysine HCl | 0.36 | 0.36 | 0.36 | 0.36 | 0.36 | 0.16 |
| DL-Methionine | 0.18 | 0.13 | 0.10 | 0.09 | 0.09 | 0.01 |
| L-Threonine | 0.15 | 0.13 | 0.12 | 0.11 | 0.11 | 0.01 |
| L-Tryptophan | 0.03 | 0.03 | 0.03 | 0.04 | 0.04 | 0.00 |
| Vitamins and microminerals* | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |
| Inert | 0.40 | 0.15 | 0.40 | 0.17 | 0.40 | 0.21 |
| **Calculated values** | | | | | | |
| ME, Kcal/kg | 3350.00 | 3350.00 | 3350.00 | 3350.00 | 3350.00 | 3350.00 |
| Crude protein, % | 20.45 | 17.86 | 15.91 | 14.29 | 14.29 | 15.09 |

| | | | | | | |
|---|---|---|---|---|---|---|
| SID[4] Lys, % | 1.23 | 1.07 | 0.95 | 0.84 | 0.84 | 0.75 |
| SID Met, % | 0.45 | 0.37 | 0.32 | 0.29 | 0.29 | 0.23 |
| SID Met + Cys, % | 0.74 | 0.63 | 0.56 | 0.51 | 0.51 | 0.46 |
| SID Thr, % | 0.80 | 0.69 | 0.61 | 0.55 | 0.55 | 0.49 |
| SID Val, % | 0.25 | 0.21 | 0.19 | 0.17 | 0.17 | 0.15 |
| SID Ile, % | 0.85 | 0.74 | 0.65 | 0.58 | 0.58 | 0.63 |
| Total calcium, % | 0.77 | 0.66 | 0.57 | 0.50 | 0.50 | 0.55 |
| Available phosphorus, % | 0.87 | 0.73 | 0.63 | 0.57 | 0.57 | 0.55 |

[*]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: 17,000,000 IU of vitamin A, 6,000,000 IU of vitamin D3, 70,000 IU of vitamin E, 6,000 mg of vitamin K3, 3,200 mg of vitamin B1, 8,500 mg of vitamin B2, 35,000 mg of vitamin pantothenic acid, 6,000 mg of vitamin B6, 44 mg of vitamin B12, 44,000 mg of nicotinic acid, 1,600 mg of folic acid, 240 mg of biotin, 600 g of choline.

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