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

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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.

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

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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 0.34m^2 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 1.15m^2 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 2 cm 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 48 h and transferred to 70% alcohol solution until the slides were prepared. The histological analyses were performed in paraffin-embedded segments, sectioned at 4 μm, 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 28 s and 18 s 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 s, 60 °C for 1 min, and 95 °C for 15 s. 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 10,000 g for 20 min at a temperature of 4 °C. 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 s 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 13,750 g 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 260 nm 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 (Degnan and Ochman, 2012).

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, 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.

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	1022	23	0.183
ADFI, g	3097	3035	3091	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	1834	1854	1845	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).

3.2. Incidence of diarrhea

As shown in Fig. 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 22nd to 42nd 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 ($P > 0.050$, Fig. 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 Fig. 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 (Fig. 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 ($P < 0.050$; Fig. 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 (Fig. 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* (Fig. 7).

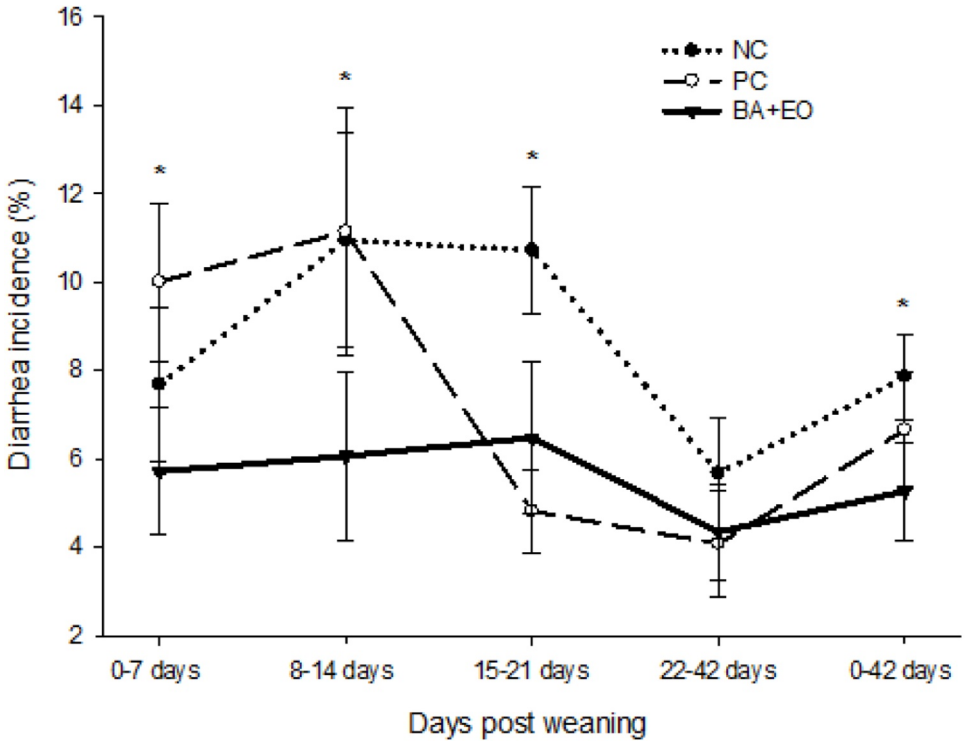

Fig. 1. Effect of experimental diets on the incidence of diarrhea in piglets in nursery phase. NC: negative control; PC: positive control (200 ppm colistin sulphate); BA + EO: supplementation with benzoic acid and essential oils (0.3%). Data are expressed as means (10 replicates/treatment) and SEM represented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

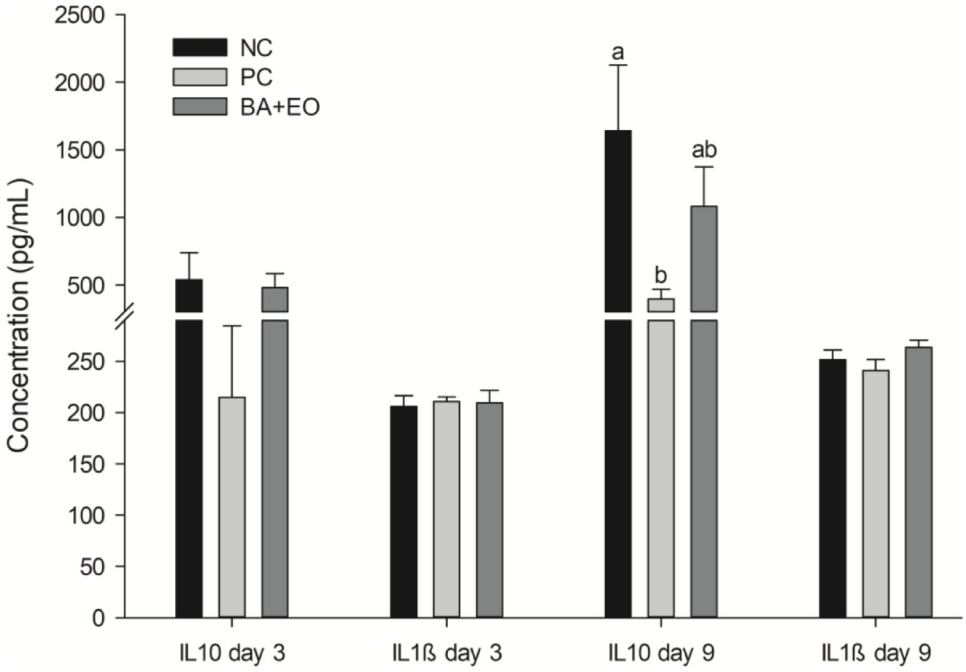

Fig. 2. Effects of experimental diets on serum levels of interleukin (IL) − 1β and IL-10 of piglets on days 3 and 9 post-weaning. NC: negative control; PC: positive control (200 ppm colistin sulphate); BA + EO: supplementation with benzoic acid and essential oils (0.3%). Data are expressed as means (5 piglets/treatment) and SEM represented by vertical bars. [ab]Significant differences between groups according to Tukey's test, $P < 0.050$.

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,

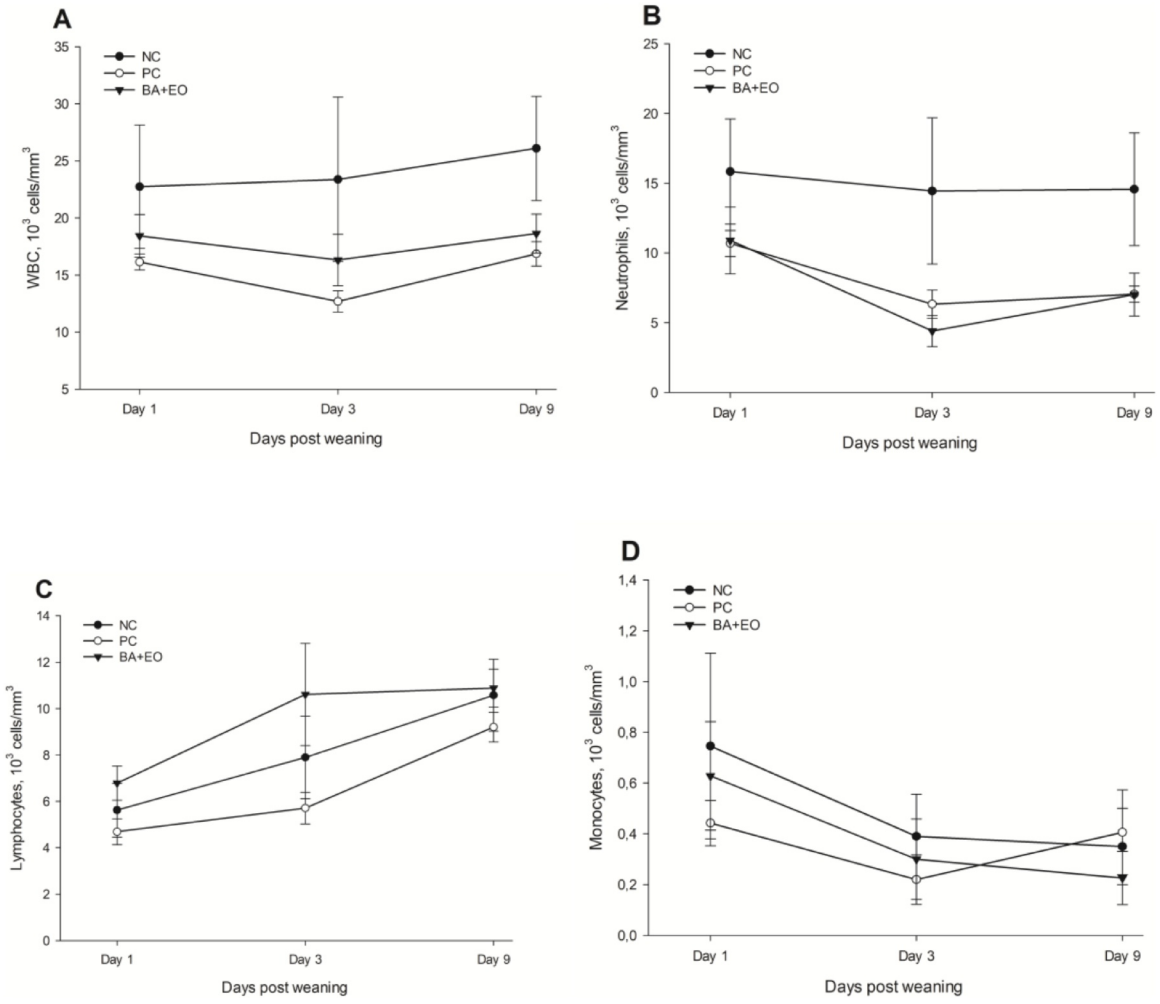

Fig. 3. Effects of experimental diets on total white blood cells (WBC) (A), neutrophils (B), lymphocytes (C), and monocytes (D) counts of weaned piglets. NC: negative control; PC: positive control (200 ppm colistin sulphate); BA + EO: supplementation with benzoic acid and essential oils (0.3%). Data 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).

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

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).

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.

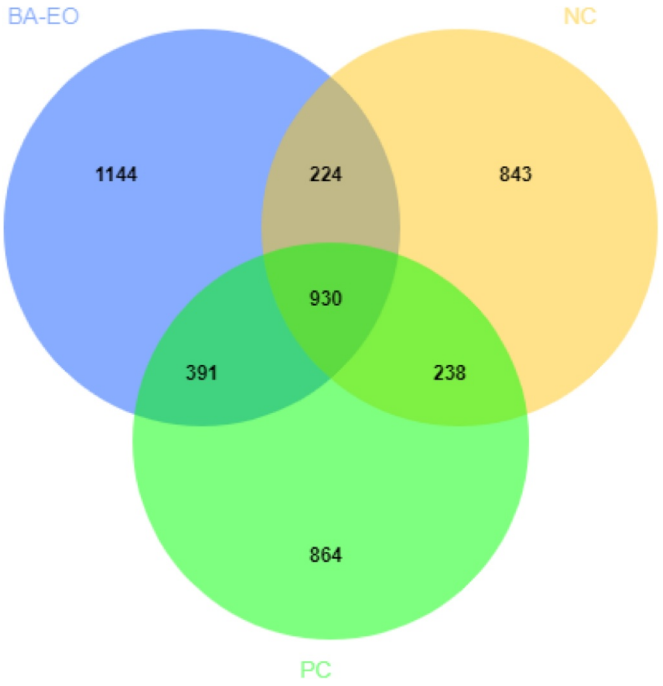

Fig. 5. Venn diagram depicting biodiversity between treatments. Values for each treatment refer to the number of OTUs observed ($P < 0.050$). NC: negative control; PC: positive control (200 ppm colistin sulphate); BA + EO: supplementation with benzoic acid and essential oils (0.3%). Piglet is an experimental unit; 7 piglets/treatment.

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).

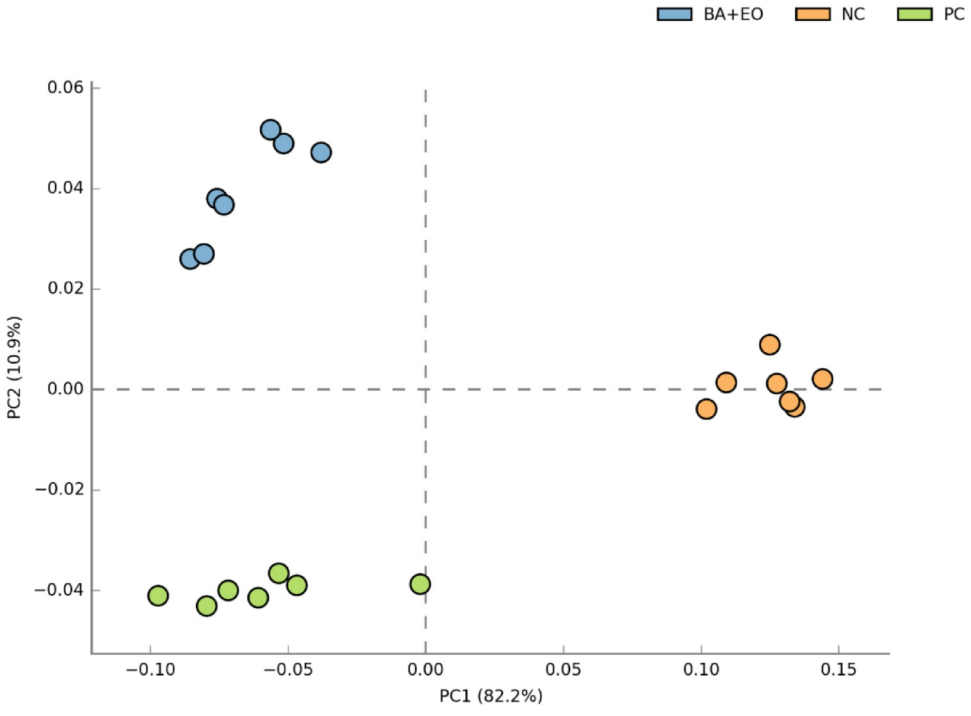

Fig. 4. Principal components analysis (PCA) of bacterial community structure between NC, PC, and BA + EO groups. Each symbol represents each gut microbiota. BA + EO: supplementation with benzoic acid and essential oils (0.3%); NC: negative control; PC: positive control (200 ppm colistin sulphate). Piglet is an experimental unit; 7 piglets/treatment.

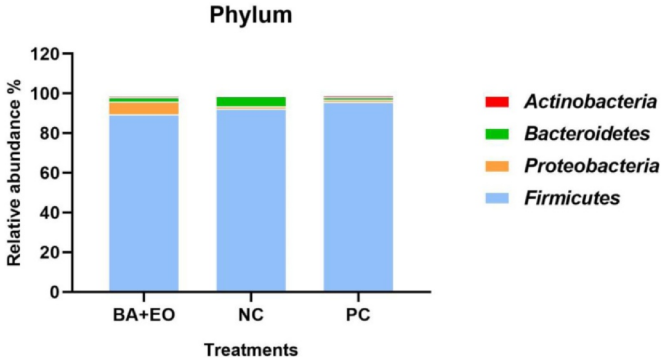

Fig. 6. Comparison of the phylum levels between treatments. BA + EO: supplementation with benzoic acid and essential oils (0.3%); NC: negative control; PC: positive control (200 ppm colistin sulphate). The phyla present differ significantly in their abundance between treatments according to the Kruskal 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%).

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, 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

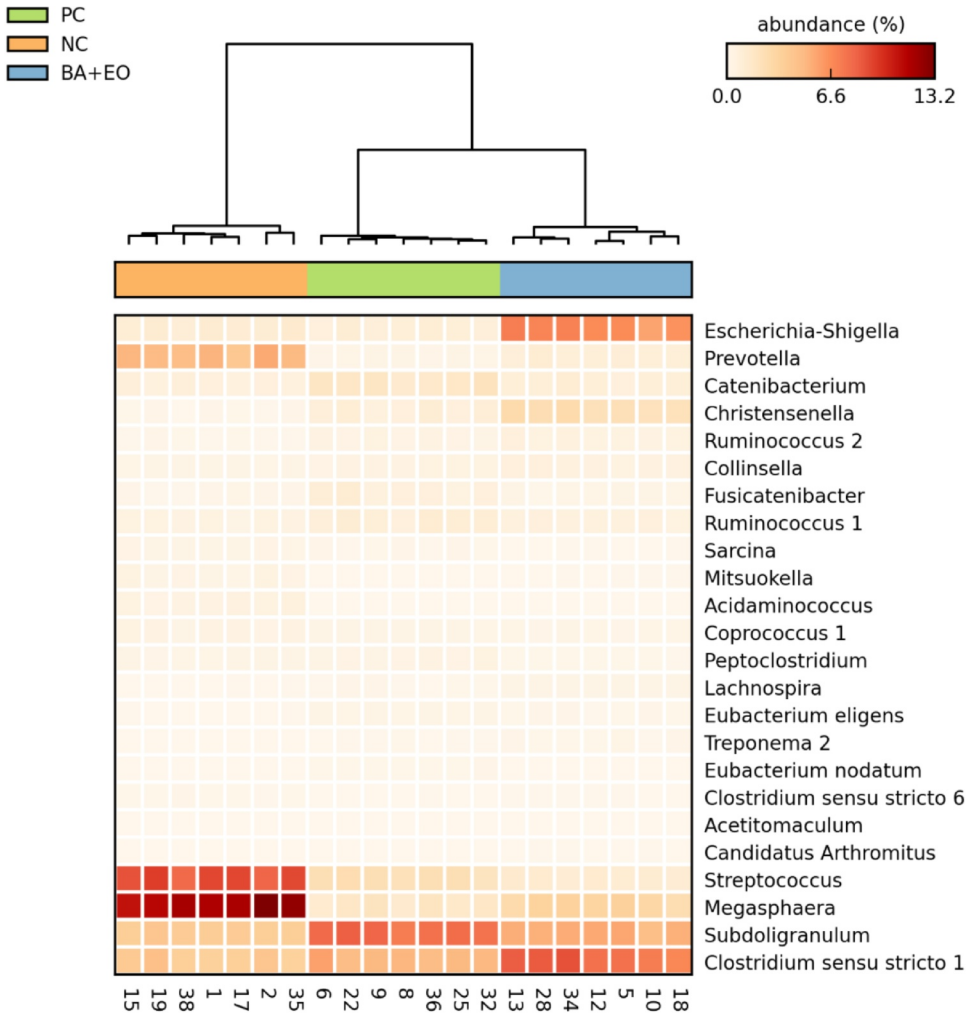

Fig. 7. Taxa that differ significantly in their abundance between treatments using the Kruskal Wallis test ($P < 0.050$). PC: positive control (200 ppm colistin sulphate); NC: negative control; BA + EO: supplementation with benzoic acid and essential oils (0.3%). Piglet is an experimental unit; 7 piglets/treatment.

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, 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.

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CRediT authorship contribution statement

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Declaration of Competing Interest

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

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2020.104311.

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