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Blend of natural and natural identical essential oil compounds as a strategy to improve the gut health of weaning pigs

D. Luise [a], F. Correa [a], C. Negrini [a], S. Virdis [a], M. Mazzoni [b], S. Dalcanale [a], P. Trevisi [a,*]

[a] Department of Agricultural and Food Sciences, University of Bologna, 40127 Bologna, Italy
[b] Department of Veterinary Science, University of Bologna, 40064 Ozzano dell'Emilia, Italy

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ABSTRACT

Weaning is one of the most critical phases in pig's life, often leading to postweaning diarrhoea (**PWD**). Zinc oxide (**ZnO**), at pharmacological doses, has been largely used to prevent PWD; however, due to antimicrobial co-resistant and environmental pollution issues, the EU banned its use in June 2022. Natural or natural identical components of essential oils and their mixture with organic acids are possible alternatives studied for their antimicrobial, anti-inflammatory and antioxidant abilities. This study aimed to evaluate the effect of two blends of natural or natural identical components of essential oils and organic acids compared to ZnO on health, performance, and gut health of weaned pigs. At weaning (d0), 96 piglets (7 058 ± 895 g) were assigned to one of four treatments balanced for BW and litter: CO (control treatment), ZnO (2 400 mg/kg ZnO from d0 to d14); Blend1 (cinnamaldehyde, ajowan and clove essential oils, 1 500 mg/kg feed); Blend2 (cinnamaldehyde, eugenol and short- and medium-chain fatty acids, 2 000 mg/kg feed). Pigs were weighed weekly until d35. Faeces were collected at d13 and d35 for microbiota (v3–v4 regions of the 16 s rRNA gene) and *Escherichia coli* (***E. coli***) count analysis. At d14 and d35, eight pigs/treatment were slaughtered; pH was recorded on intestinal contents and jejunal samples were collected for morphological and gene expression analysis. From d7–d14, the Blend2 had a lower average daily gain (**ADG**) than CO and ZnO ($P < 0.05$). ZnO and Blend1 never differed in ADG and feed intake. At d14, ZnO had a lower caecum pH than all other treatments. The CO treatment had a higher abundance of haemolytic *E. coli* than Blend1 ($P = 0.01$). At d13, the ZnO treatment had a lower alpha diversity ($P < 0.01$) and a different microbial beta diversity ($P < 0.001$) compared to the other treatments. At d13, the ZnO treatment was characterised by a higher abundance of Prevotellaceae_NK3B31_group (Linear Discriminant Analysis (**LDA**) score = 4.5, $P = 0.011$), *Parabacteroides* (LDA score = 4.5, P adj. = 0.005), the CO was characterised by Oscillospiraceae UCG-005 (LDA score = 4.3, P adj. = 0.005), Oscillospiraceae NK4A214_group (LDA score = 4.2, P adj. = 0.02), the Blend2 was characterised by *Megasphaera* (LDA score = 4.1, P adj. = 0.045), and *Ruminococcus* (LDA score = 3.9, P adj. = 0.015) and the Blend1 was characterised by Christensenellaceae_R-7_group (LDA score = 4.6, P adj. < 0.001) and *Treponema* (LDA score = 4.5, P adj. < 0.001). In conclusion, Blend1 allowed to maintain the gut health of postweaning piglets through modulation of the gut microbiome, the reduction of haemolytic *E. coli* while Blend2 did not help piglets.

Implications

Feeding strategies aimed at replacing the pharmacological dose of zinc oxide is a priority for pig farming. Natural and natural identical essential oil compounds could represent a potential strategy. The results demonstrated that the blend composed by cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), aiowan essential oil (titrated in thymol) and essential oil of clove (titrated in eugenol) reduced the concentration of potential intestinal pathogens typical of the postweaning period such as enterotoxigenic *Escherichia coli* and increase the abundance of short-chain fatty acid-producing bacteria in postweaning piglets. These effects may indicate a possible improvement in resilience and the promotion of gut health.

Introduction

Weaning is always accompanied by changes in the functionality, intestinal morphology and of gut microbial ecosystem, and in

* Corresponding author.
E-mail address: paolo.trevisi@unibo.it (P. Trevisi).

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specific conditions, it can predispose the piglets to postweaning diarrhoea (**PWD**). Generally, PWD is recognised as a multifactorial event in which social, environmental and physiological factors contribute to the development of dysbiosis resulting in an increase in piglets' morbidity, mortality, antibiotic use and treatment costs (Luppi, 2017). Dietary zinc oxide (**ZnO**) at a pharmacological dose (2 500 mg/kg) has largely been used as an effective alternative to antibiotics in the postweaning phase to reduce antibiotic use and the incidence of PWD. However, since it has been observed a strong correlation between high levels of ZnO and environmental issues, as well as the selection of antibiotic-resistant bacteria, the European Commission has decided to ban the use of pharmacological levels of ZnO since 2022 (European Medicines Agency, 2017). In this regard, new strategies to face the problems related to the weaning transition are needed. Among these, feeding strategies are deeply studied and the use of natural and natural identical essential oil compounds has shown promising results. A good number of studies, on the usage of natural and natural identical essential oil compounds on weaned piglets, are reported and the most used bioactive substances are carvacrol, eugenol, cinnamaldehyde, thymol, and capsaicin (Huang and Lee, 2018). Natural and natural identical essential oil compounds used can result in a reduction of inflammatory processes, improved digestibility of DM and CP of the diet, enhanced growth performances, and improved gut morphology (Huang and Lee, 2018). On the contrary, it has also been outlined that natural and natural identical essential oil compounds could have no effect on nutrient digestibility, growth performance, feed intake, villus height of the intestinal epithelium and gut microbial composition (Huang and Lee, 2018). Given the lack of consistency found in the literature which could be due to the different formulation of the tested products, it is necessary to increase the number of experiments aimed at testing the effectiveness of these products as well as create specific data on each formulation. In particular, cinnamaldehyde has a bactericidal effect against *Escherichia coli* (***E. coli***), interfering with the synthesis of DNA, RNA, proteins and glucosamine (Pereira et al., 2021). Thymol and carvacrol have known bactericidal activity against *E. coli* linked to the lysis of the bacterial cytoplasmic membrane (Xu et al., 2008). Eugenol has been shown to have a synergistic bacteriostatic effect against *E. coli* when combined with cinnamaldehyde, thymol and carvacrol (Pei et al., 2009). Furthermore, the combination of carvacrol, thymol and cinnamaldehyde has shown to be effective in maintaining the antioxidant balance and reducing gut inflammation in postweaning pigs (Rebucci et al., 2022). Therefore, it was hypothesised that the administration of a mixture of natural or natural identical components of essential oils based mainly on cinnamaldehyde, ajowan, clove and eugenol could reduce the abundance of pathogens, including the *E. coli* and improve intestinal health of postweaning piglets.

As an additional strategy to prevent the use of antibiotics and replace the high dose of ZnO in postweaning period, organic acids have also been widely tested. Propionic acid, formic acid, butyric acids, and mono and diglycerides of fatty acids have been reported to have a positive effect on controlling PWD, increasing the performance and reducing the proliferation of the pathogens in the intestine of piglets (Correa et al., 2021). Since the organic acids and the natural and natural identical essential oils have different modes of action, it is possible that their combination could be synergistic, resulting in a broader spectrum of activities (Langhout, 2020). Indeed, organic acids are shown to exert their activity mainly in the upper part of the gastrointestinal tract, while encapsulated natural and natural identical essential oils and encapsulated organic acids can act in the distal part of the intestine (Langhout, 2020). For instance, according to the study of Rodrigues et al. (2020), the supplementation of thymol, 2-methoxyphenol, eugenol, piperine, and curcumin components in combination with benzoic acid (3 g/kg in total) improved the growth performance of weaned piglets compared to a normal diet having better response compared with benzonic acid alone. On the contrary, the study of Zhai et al. (2020) suggested that the supplementation of 2 g/kg essential oil compounds (main ingredients: thymol, eugenol, and piperine) plus benzoic acid had comparable results to the supplemental of benzoic acid alone. In addition, both studies suggested a significant effect in the response due to the dose of integration (Rodrigues et al., 2020; Zhai et al., 2020). In addition, to date, literature testing the effect of mixture of natural and natural identical essential oils and organic acids as a potential strategy to replace the use of a high dose of ZnO in weaned piglets is still limited.

Considering what has been reported, the hypothesis underlying this study is that the use of mixtures of natural and natural identical essential oils alone and mixed with organic acids could be a good strategy to replace ZnO at pharmacological doses in postweaning piglets thanks to their different modes of action which includes antibacterial, anti-inflammatory and oxidative regulation activities.

Following our preliminary results (Correa et al., 2023), the present study aimed to evaluate the effect of the administration and the modes of action of two blends of natural and natural identical essential oils containing mainly cinnamaldehyde, thymol, carvacrol and eugenol alone or in combination with a mixture of organic acids compared to the pharmacological dose of ZnO on the health, growth performance, and gut health, including faecal microbial profile of weaned pigs.

Material and methods

Experimental design and sampling

At weaning (26.53 ± 0.5 days of age – d0), a total of 96 piglets with an average BW (7 058 ± 895 g) were selected from nine sows (12 piglets from six sows and eight piglets from three sows) previously tested for being genetically susceptible to *E. coli* F18 (Luise et al., 2019a). Indeed, the genotypes derived from nucleotide polymorphism located on Fucosyltransferase 1 (***FUT1***) gene, which has been associated with the susceptibility of piglets to enterotoxigenic *E. coli* carrying the adhesive fimbriae F18, can affect the metabolism and microbiome of postweaning piglets (Luise et al., 2019a). At d0, piglets were moved to the experimental facility of the University of Bologna and located in pens. Each pen was equipped with enrichment materials (a chain and a natural cotton rope). The room temperature and humidity were recorded daily. The temperature was set to decrease gradually from 30 °C at d0 to 28 °C at d35, and extra heating was provided by infrared lamps for the first 7 days postweaning to ensure a thermal comfort area in the pen.

At d0, the individual BW was recorded and piglets were assigned to one of the four dietary treatments balanced for their BW and litter of origin. Each dietary treatment had eight replicates, with three piglets per pen.

Bristles from piglets were collected at d0 to genotype the animals for susceptibility for *E.coli* F18 (Luise et al., 2019b). Piglets were individually weighted at d0, d7, d14, d28 and d35 (end of the study). Feed intake was recorded daily. To evaluate the piglets' gut health status, the individual faecal score was daily recorded by visual appraisal using a five-point scoring system (1–5): from 1: hard to 5: watery faeces, where a faecal score >3 was considered as diarrhoea (Luise et al., 2019b). The mean of the faecal score was calculated individually for each period.

Faecal samples were collected at d13 from 16 piglets per diet (2 piglets per pen with an average daily gain (**ADG**) representative of the average ADG of the treatment), and at d34 from eight piglets

per diet (one piglet per pen with an ADG representative of the average ADG of the treatment) into a sterile tube. Faecal samples were snap frozen in liquid nitrogen and stored a – 80 °C for further microbiota analysis using the next-generation sequencing approach. Moreover, at d13, from a subgroup of eight piglets per diet (1 piglet per pen, chosen from the 16 previously selected for faecal sampling), 5 g of fresh faecal samples were used to determine the concentration of total *E. coli* and haemolytic *E. coli* by plate counts.

Serum samples were collected at d14 and d35 from eight piglets (same piglets sampled for the microbiota composition) per diet by venipuncture of the vena cava using a collection tube with a clot activator (Vacutest Kima Srl, Arzergrande, PD, Italy). To obtain serum, blood was incubated at room temperature for 2 h, then centrifuged at 3 000*g* for 10 mins (Luise et al., 2019b). Serum was then stored at −20 °C until reactive oxygen metabolites (**ROMs**) analysis.

On d14 and d35, eight piglets per diet (1 piglet per pen, the same sampled for the *E. coli* counting at d13 and for microbiota composition at d34) per time-point were killed with an intracardiac injection of Tanax® (embutramide 200 mg/mL, mebenzonium iodide 50 mg/ml tetracaine hydrochloride 5 mg/mL and 5 mL/kg BW; Intervet Productions srl, Aprilia, Italy) after the anaesthesia with Zoletil 100 (Virbac, Milano, Italy; 15 mg/kg BW). At d14 and d35, after the piglets' sacrifice, the gut was immediately removed. The mucosa from the distal part of the jejunum (75% of the small intestine length) was gently scraped and snap-frozen in liquid nitrogen and then preserved at −80 °C for gene expression analysis. Furthermore, an additional jejunum sample was collected for morphometric analysis. For the morphometric analysis, tissue was fixed overnight in neutral buffered formalin at 10%, embedded in paraffin and then retained in haematoxylin-eosin for morphological analysis. Five g of jejunal, cecal and colonic contents were collected to measure the pH using a pH meter (Vio 7, Sinergica Soluzioni, Milan, Italy).

Diet composition

Two-phase diets were used during the experiment; phase 1 from d0 to d14, and phase 2 from d15 to d35. The four dietary treatments were: (1) basal diet commonly used at weaning without antimicrobials and pharmacological level of ZnO (CO); (2) the same diet of the CO treatment supplemented with ZnO at a pharmacological dose (2 400 mg/kg) only from d0 to d14 (ZnO); (3) same diet of the CO treatment but integrated with the Blend1 at 1 500 mg/kg fed from d0 to d35; (4) same diet of the CO treatment but integrated with Blend2 at 2 000 mg/kg of fed from d0 to d35. ZnO, Blend1 and Blend2 supplements were included on top to the CO diet. Blend1 and Blend2 were commercial product provided by Chemirfarma Spa (Forli, Italy).

Blend1 was composed of cinnamaldehyde (naturally identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol) (Chemifarma Spa, Forlì, Italy). The Blend1 was composed of a total extract of 85.2 g/kg including 40 g/kg of cinnamaldehyde, 10.65 g/kg of thymol, 9.84 g/kg of carvacrol, 8.31 g/kg of p-Cymene, 8.06 g/kg of eugenol, 4.57 g/kg of y-Terpinene, 1.91 g/kg diallyl disulphide and 1.88 g/kg beta-caryophyllene and a matrix of 14.78 g/kg composed of wheat middling, calcium carbonate and corn starch. The product was microencapsulated using a patented system that uses natural polysaccharides.

Blend2 was composed of fatty acids in ester form, eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), and methyl salicylate (synthetic). The total extract was 100 g/kg of the product including 88 g/kg of cinnamaldehyde, 9 g/kg of eugenol and 3 g/kg of methyl salicylate. The mono and diglycerides of fatty acids were 400 g/kg including, in order of concentration, butyric acid esters, propionic acid esters, capric and caprylic acid esters, free glycerol, free fatty acids and water. The product was microencapsulated using 340 g of refined and hydrogenated palm oil and 160 g of silicic acid.

The natural identical compounds were produced synthetically while the natural essential oil components were extracted with steam distillation from plants.

The composition of the CO diet and its relative calculated chemical analysis is shown in Table 1. All diets were formulated to meet or exceed the nutrient requirements of weaned piglets suggested by NRC (2012). Piglets had free access to water and feed during the entire duration of the trial.

Animal genotyping

The genomic DNA of each piglet and sow was extracted from bristles according to the protocol of Luise et al. (2019b). Samples were stored at −20 °C. Genotyping of *FUT1* g.307 G > A was carried out by using the PACE™ Genotyping approach using the genotyping assay previously reported by Luise et al. (2019b) using the QuantStudio 7 Instrument (Applied BioSystems, Foster City, USA).

Microbial analysis

Escherichia coli enumeration

One g of the sample was weighed into a sterile tube and then suspended in 9 mL of a Buffered Peptone Water (Oxoid; Hampshire, UK). The suspension was vortexed until the sample was completely homogenised. Afterwards, the suspension was 10-fold serially diluted (from 10-1 up to 10-8) in Buffered Peptone Water (Oxoid). A volume of 0.1 ml of each serial dilution was then plated onto each medium. For the enumeration of *E. coli*, Triptone-bile-glucuronide (Oxoid) medium plates were incubated aerobically for 24 h at 44 °C. Haemolytic bacteria were enumerated on blood agar (Oxoid Pb5039A) after aerobic incubation for 1 day followed by a confirmation using Tryptone Bile X glucoronide Agar (Oxoid). After incubation, the total number of *E. coli* and haemolytic *E. coli* was counted from plates with 30 to 150 colonies.

Microbial profile

Total bacterial DNA was extracted from faeces using FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The V3-V4 hypervariable regions of the 16S rRNA gene amplicons were amplified using the Pro341F and Pro805R primers (Luise et al., 2022). The standard protocol for MiSeq Reagent Kit v3 was used to prepare the libraries which were sequenced on MiSeq platform (Illumina Inc., San Diego, CA, USA). Microbiota bioinformatics analysis was performed using the DADA2 pipeline (Callahan et al., 2016), and taxonomy was assigned using Silva Database (release 138; Quast et al., 2012).

Morphometric analysis

Formalin-fixed, paraffin wax-embedded 7 µm thick sections were deparaffinised in xylene and stained with haematoxylin–eosin. At least 10 sections were obtained from each paraffin-embedded sample and mounted on poly-L-lysinecoated slides. For each sample, the height, and the width of 20 villi, and the width and the depth of 20 crypts were measured. The sections were examined at low magnification with a conventional microscope interfaced with a digital camera and a personal computer equipped with Cytometry software (Byk Gulden, Milan, Italy). The mucosal-to-serosal amplification ratio, representing the absorptive mucosal surface in the jejunum, was calculated as

Table 1
Ingredients and calculated composition of phase I and phase II basal diet of postweaning piglets.

Items	Phase I, d0–d14	Phase II, d14–d35
Ingredients, %		
Barley flour	20	15
Barley PS70 piglets	18	17.64
Common wheat	10.1	8.2
Wheat middlings	9.91	10
Soybean meal, 48%	8	11.8
Oat, decorticated and flaked	5	-
Corn	5	15
Lactose	4	3
Soybean microflaked	4	2.5
Common wheat bran	4	5.9
Pig plasma	3	–
Soybean oil	1.5	2.1
Fishmeal		2.1
Dicalcium phosphate	1	1
Beet pulp	1	1.5
Potato protein	1	-
Fibre concentrate	1	1
Coconut oil	0.8	0.5
L-Lysine HCL	0.59	0.59
Piglets 604 Enzymes mix 0.4%[1]	0.4	0.4
Sodium Chloride	0.4	0.4
Calcium formate	0.3	0.3
L-Threonine	0.23	0.25
DL-Methionine	0.21	0.22
Citric Acid	0.2	0.2
Val-Ile-Leu-His Premix	0.2	0.25
Aromatic compounds	0.07	0.07
L-Tryptophan	0.07	0.06
Vitamin and mineral Premix[2]	0.03	0.03
Calculated chemical composition, fed basis		
DM	89.69	89.29
Water	10.31	10.71
CP	16.77	16.03
Crude Lipid	5.54	5.55
Crude Fibre	3.45	3.6
Ash	4.1	4.33
NDF	13.64	14.11
ADF	4.54	4.8
ADL	0.83	0.79
Starch	40.71	40.27
Sugar	7.05	6.35
Lysine	1.31	1.20
Methionine	0.45	0.46
Met + Cyst	0.78	0.72
Tryptophane	0.29	0.26
Threonine	0.86	0.79
Leucine	1.3	1.22
Isoleucine	0.64	0.64
Valine	0.9	0.83
Arginine	0.95	0.91
Calcium	0.46	0.52
Phosphorus	0.54	0.58

[1] Piglets 604 Enzymes mix 0.4%: phytase (375.000 FYT/kg), Endo 1,4 beta glucanase (20.040 U/kg), Endo 1,4 beta xilanase (67.635 U/kg), Endo 1,3(4) beta glucanase (17.535 U/kg).
[2] Vitamins and mineral premix: Vitamin A 15 000.00 UI, Vitamin D3 1 800.00 UI, Betaine hydrochloride 180.00 mg, Biotin 0.15 mg, Choline chloride 94.50 mg, Folic acid 0.99 mg, Niacin 72.00 mg, D-pantothenate Calcium 13.33 mg, Vitamin B1 5.10 mg, Vitamin B2 9.90 mg, Vitamin B6 6.00 mg, Vitamin E 102.00 mg, Vitamin K3 2.49 mg, Copper 99.60 mg, Iodine 1.50 mg, Iron 120.00 mg, Manganese 75.00 mg, Selenium 0.30 mg, Zinc 99.60 mg.

$$\text{mucosal} - \text{to} - \text{serosal amplification ratio} = (\text{villous surface} + \text{unit bottom} - \text{villous bottom})/\text{unit bottom}$$

As previously reported by Trevisi et al. (2009) where villous surface: π (villous length × villous width), unit bottom = π (villous width/2 + crypt width/2)^2 and villous bottom = π (villous width/2)^2.

Reactive oxygen metabolites and gene expression analysis

The serum was analysed in duplicate for reactive oxygen metabolites colorimetrically using the d-reactive oxygen metabolites test kit (Diacron International Sr1, Grosseto, Italy) following the procedure described by Luise et al. (2022). Total RNA was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and treated with TURBO DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) to remove contaminating DNA following the protocol. The High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) was used to convert a total of 800 ng of RNA into complementary DNA. Duplex Real-Time PCR reactions were run in triplicate on the Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR system (Thermo Fisher Scientific) following the thermocycler settings described by Luise et al. (2022) for the genes reported in Supplementary Table S1 and using Hydroxymethylbilane synthase gene as housekeeping gene (Luise et al., 2019b). The QuantStudio Design and Analysis Software v2.5 (ThermoFisher Scientific, Waltham, MA, USA) was used for determining the values of threshold cycle of the genes, and the expression of the target genes was given using the $2^{-\Delta\Delta}$ cycle threshold method.

Statistical analysis

Data of BW and ADG were fitted using a linear mixed model in which the diet was included as a fixed factor and litter of origin and pen were included as random factors. Data on morphometric measures, pH, ROMs, and gene expression were fitted using a linear mixed model in which the diet was included as a fixed factor and litter of origin was included as random factor. Sex and genotype for *FUT1* were initially included as factors but then removed as they were not significant. BW at d14 and d35 was included as covariate for morphometric measures, ROM parameters, and gene expression and removed when not significant.

For feed intake (**FI**) and feed-to-gain ratio (**F:G**), the pen was used as an experimental unit and data were fitted using a linear model including diet as a fixed factor. Data on bacterial counts were fitted using a general linear model (using the Gaussian distribution for the total *E. coli* and the quasipoisson for the hemolytic *E. coli*) including the diet as a fixed factor after Log transformation. The differences among treatments were determined by using the Tukey multiple comparison of means test.

The packages "car" (version 3.0.12), "lm4" (version 1.1.27.1) and "lsmeans" (version 2.30) within the R software (R Core Team, 2021) were used. *P*-value ≤0.05 was considered significant, and *P*-value <0.1 was considered a trend of significance.

Regarding the microbial data, the statistical analysis on alpha diversity and beta diversity and taxonomics was carried out with R v4.1, using "phyloseq" (McMurdie and Holmes, 2013) v1.38, "vegan" v2.6 (Dixon, 2003) and "microbiomeutilities" v1.0 (https://github.com/microsud/microbiomeutilities). For the alpha diversity, samples were rarefied to the lowest sample depth, to avoid bias linked to different sampling efforts. Differences in alpha diversity indices (Chao1, Shannon, and Simpson diversity) between experimental diets were tested using a Wilcoxon test. For the beta diversity, a dissimilarity matrix using Euclidean distances of centred log-ratio transformed data was constructed and results were plotted using a Principal Coordinates Analysis plot. Differences were tested using a PERMANOVA model (Adonis test) with 9 999 permutations, including diet as a factor. Pairwise contrasts were carried out using pairwiseAdonis function included in the "PairwiseAdonis" package v0.4 package (https://github.com/pmartinezarbizu/pairwiseAdonis). *P*-values were then adjusted for multiple comparisons using Benjamini-Hochberg correction. Previous to the adonis test, we assessed the homogeneity of the

dispersion between treatments. Linear discriminant analysis (**LDA**) effect size algorithm at Genus levels was applied to identify taxa differentially expressed (LDA score >3 and *P*. adj < 0.05) between the dietary treatments.

The R scripts used to carry out the statistical analysis are reported in Supplementary Material section.

Results

Health and performance

For *FUT1*, 31 pigs $FUT1^{GG}$, 56 pigs $FUT1^{AG}$ and nine pigs $FUT1^{AA}$ were observed, where the $FUT1^{AA}$ is the resistant genotype for enterotoxigenic *E. coli* F18.

During the trial, one piglet of Blend1 treatment was excluded from the trial for health impairment due to *Streptococcus suis* infection at d2. At d7 two piglets, respectively from ZnO and Blend2 treatments, and one piglet of the Blend1 treatment at d14 were treated for health impairment. These piglets were excluded from the choice of sampling.

The other piglets stayed healthy until the end of the study. The results of the weekly faecal score are shown in Supplementary Table S2. No difference among treatments were observed.

Table 2 reports the effect of the diet on the BW, ADG, FI and F:G ratio of piglets. No significant difference in BW between treatments at any timepoint was observed.

No significant differences between the treatments were observed for the ADG in the periods d0–d7, d14–d28, d28–d35, d14–d35 and d0–d35. From d7–d14 and from d0–d14, the diet significantly affected the ADG ($P < 0.0001$ and $P = 0.002$ respectively); the CO and ZnO treatments had a higher ADG compared with the Blend2 treatment ($P < 0.05$) with intermediate value observed for Blend1.

No effect of the diet was observed in the FI for the periods d0–d7, d14–d28 and d28–d35. From d7 to d14 and from d0 to d14, the diet significantly affected the FI ($P = 0.05$); from d7–d14, the CO treatment had a higher FI compared with the Blend2 treatment ($P = 0.04$) while no difference was observed between the other treatments; from d0–d14, the CO and the Blend1 treatments tended to have a higher FI compared with the Blend2 treatment ($P = 0.09$ and $P = 0.06$, respectively) while no differences were observed between the other treatments.

Regarding the F:G ratio, no effect of the diet was observed for the periods d0 – d7, d14–d28, d28–d35, d14–d35 and the total period. From d7 to d14 and from d0 to d14, the diet significantly affected the F:G ($P = 0.01$); from d7–d14, the CO and ZnO treatments had a lower F:G compared with the Blend2 treatment ($P = 0.04$; $P = 0.03$; respectively) while the Blend1 has intermediate value; from d0 – d14, the CO and ZnO treatments had a lower F:G compared with the Blend2 treatment ($P = 0.01$; $P = 0.02$, respectively) and the CO treatment tended to have a lower F:G compared with the Blend1 treatment ($P = 0.09$) while no differences were observed between Blend1 and ZnO and Blend1 and Blend2.

Intestinal pH and microbial profile

The results of the pH and faecal count are reported in Table 3. No effect of the diet was observed in the pH of the jejunum at

Table 2
Effect of supplementation with Blend1 and Blend2 on live weight, daily weight gain, feed intake and feed-to-gain ratio of postweaning piglets.

Items	Diet[1]				SEM	*P*-value
	CO	ZnO	Blend1	Blend2		
BW, g						
d0	7 077	7 066	7 077	7 041	325	1.00
d7	7 811	7 762	7 970	7 660	341	0.93
d14	10 336	10 124	10 003	9 346	403	0.31
d28	17 720	17 433	17 243	16 144	587	0.13
d35	22 495	22 235	22 235	20 541	722	0.12
Average daily gain, g/d						
d0–d7	104.2	99.0	125.4	91.4	17.7	0.45
d7–d14	361^{A}	337^{A}	290^{AB}	240^{B}	26.7	<0.0001
d0–d14	233^{A}	218^{A}	208^{AB}	165^{B}	14.1	0.002
d14–d28	520	517	531	484	22.8	0.38
d28–d35	682	682	716	630	32.5	0.31
d14–d35	574	574	592	532	22.7	0.20
d0–d35	438	432	431	387	17.7	0.10
Feed intake, g/d						
d0–d7	137	142	166	128	14.5	0.32
d7–d14	467^{A}	436^{AB}	448^{AB}	364^{B}	25.8	0.05
d0–d14	302^{a}	289^{ab}	307^{a}	246^{b}	16.5	0.05
d14–d28	813	787	811	714	34.2	0.17
d28–d35	1 135	1 172	1 184	1 065	50.0	0.35
d14–d35	921	915	935	831	35.7	0.18
d0–d35	673	665	684^{a}	597^{b}	25.5	0.09
Feed to gain						
d0–d7	1.37	1.59	1.33	1.47	0.10	0.27
d7–d14	1.30^{A}	1.29^{A}	1.56^{AB}	1.66^{B}	0.08	0.01
d0–d14	1.30^{A}	$1.32^{A,b}$	$1.47^{b,C}$	1.53^{C}	0.05	0.01
d14–d28	1.57	1.53	1.52	1.49	0.04	0.43
d28–d35	1.67	1.72	1.64	1.70	0.03	0.38
d14–d35	1.61	1.60	1.57	1.57	0.03	0.55
d0–d35	1.54	1.54	1.57	1.55	0.02	0.82

[1] Diet: CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic). Different capital letters indicate significant differences between treatments (*P*-value < 0.05), while lowercase letters indicate a trend of significance between treatments.

Table 3
Effect of supplementation with Blend1 and Blend2 on gut pH and faecal bacterial count of postweaning piglets.

Items	Diet[1]				SEM	*P*-value
	CO	ZnO	Blend1	Blend2		
pH d14						
Jejunum	7.06	7.31	6.99	7.22	0.13	0.33
Caecum	6.13^{A}	6.65^{B}	5.82^{A}	5.84^{A}	0.10	<0.0001
Colon	6.43	6.79^{b}	6.46	6.35^{a}	0.12	0.06
pH d35						
Jejunum	7.49	7.60	7.60	7.40	0.11	0.53
Caecum	6.04	6.27	6.01	5.99	0.11	0.24
Colon	6.51	6.33	6.44	6.42	0.13	0.79
Faceal bacterial count d13, Log						
E. coli	5.27	4.97	4.56	5.40	0.40	0.47
Haemolytic *E. coli*	2.64^{A}	2.25^{AB}	2.02^{B}	2.46^{AB}	0.16	0.05

Diet[1]: CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic). Different capital letters indicate significant differences between treatments (*P*-value < 0.05), while lowercase letters indicate a trend of significance between treatments.

d14 and the pH of the jejunum, cecum and colon at d35. At d14, the diet significantly affected the pH of the cecum ($P < 0.0001$); the ZnO had a higher pH compared with the other dietary treatments ($P < 0.009$). Same tendency was observed for the pH of the colon ($P = 0.06$) at d14.

At d13, no difference in the faecal abundance of total *E. coli* was observed between treatments, while the faecal abundance of haemolytic *E. coli* was significantly influenced by the diet ($P = 0.05$); the CO treatment had a higher abundance of haemolytic *E. coli* compared with the Blend1 treatment ($P = 0.04$) while ZnO and Blend2 treatments had intermediate values (Table 3).

Bacterial DNA from faecal samples was successfully extracted and amplified from a total of 95 samples. Overall, the sequencing procedure produced a total of 7 593 988 sequences, with an average of 79 104 per sample, after quality check, an average of 43 804 reads per sample were retained. After the bioinformatic analysis, a total of 6 654 Amplicon Sequence Variants were produced. Rarefaction curves are reported in Supplementary Fig. S1, which shows the number of different species observed as a function of the number of sequences; the tendency to a plateau of the curves indicates that the sequencing procedure was able to capture all the variability present in the samples.

Among the 6 654 Amplicon Sequence Variants recovered, 23 Phyla, 121 Families and 293 Genera were identified. The most abundant phyla (mean and SD) were Firmicutes 67.4 ± 11.3%, Bacteroidota 25.5 ± 10.0% and Spirochaetota 3.1 ± 4.6%. The most abundant families were Lachnospiraceae 17.7 ± 8.3%, Prevotellaceae 14.1 ± 7.8%, Lactobacillaceae 9.8 ± 9.7% and Oscillospiraceae 8.6 ± 8.0%. The most represented genera were *Lactobacillus* 9.8 ± 9.7%, *Prevotella* 6.3 ± 6.0%, Prevotellaceae_NK3B31_group 4.6 ± 4.5% and *Clostridium_sensu_stricto*_1 4.4 ± 6.3%.

Fig. 1 shows the values for Chao1, Shannon and InvSimpson diversity indices for each treatment at d13 and d34. At d13, the ZnO treatment had a significantly lower alpha diversity, for all the indices considered, compared with the CO treatment (Chao1, $P = 0.001$; Shannon, $P = 0.0006$; InvSimpson, $P = 0.0058$), the Blend1 treatment (Chao1, $P < 0.0001$; Shannon, $P = 0.0001$; InvSimpson, $P = 0.0014$) and the Blend2 treatment (Chao1, $P < 0.0001$; Shannon, $P = 0.0001$; InvSimpson, $P = 0.0026$). At d34, no difference between the treatments was observed.

The betadisper analysis showed no differences in the homogeneity of the treatments. Results of the Principal Coordinates Analysis plot for the beta diversity are shown in Fig. 2A and B for d13 and d34, respectively. The Adonis test showed that the bacterial structure was significantly affected by diet at d13 ($R^2 = 0.1$, $P = 0.001$). The pairwise contrast showed that the ZnO treatment had a significantly different bacterial composition compared with the CO ($R^2 = 0.09$, P adj. < 0.001), Blend1 ($R^2 = 0.11$, P adj. < 0.001) and Blend2 ($R^2 = 0.11$, P adj. < 0.001) treatments. At d34, the bacterial structure continued to be affected by the diet ($R^2 = 0.18$, $P = 0.046$); however, the differences among the treatments were mostly driven by the comparison between ZnO and Blend1 ($R^2 = 0.1$, P adj. = 0.01) and no differences were observed for the other comparisons.

To identify specific bacterial markers which were differentially expressed between treatments, the LDA effect size analysis was conducted and results are reported in Fig. 3A and B for d13 and d34, respectively. At d13, the ZnO treatment was characterised by a higher abundance of Prevotellaceae_NK3B31_group (Linear Discriminant Analysis (**LDA**) score = 4.5, P adj. = 0.011), *Parabacteroides* (LDA score = 4.5, P adj. = 0.005), *Bacteroides* (LDA score = 4.4, P adj. < 0.001) and *Intestinibacter* (LDA score = 4.1, P adj. = 0.003). The CO treatment was characterised by a higher abundance of Oscillospiraceae_UCG-005 (LDA score = 4.3, P adj. = 0.005), Oscillospiraceae_NK4A214_group (LDA score = 4.2, P adj. = 0.02), *Faecalibacterium* (LDA score = 3.7, P adj. = 0.047), *Solobacterium* (LDA score = 3.7, P adj. < 0.001), Marvinbryantia (LDA score = 3.7, P adj. < 0.001), Lachnospiraceae_XPB1014_group (LDA score = 3.7, P adj. < 0.001), *Catenibacterium* (LDA score = 3.6, P adj. = 0.049) and *Fusicatenibacter* (LDA score = 3.5, P adj. = 0.004). The Blend1 group was characterised by a higher abundance of Megasphaera (LDA score = 4.1, P adj. = 0.045), Anaerostipes (LDA score = 3.9, P adj. = 0.001), *Ruminococcus* (LDA score = 3.9, P adj. = 0.015), Acetitomaculum (LDA score = 3.7, P adj. < 0.001). The Blend2 treatment was characterised by a higher abundance of Christensenellaceae_R-7_group (LDA score = 4.6, P adj. < 0.001), *Treponema* (LDA score = 4.5, P adj. < 0.001), Oscillospiraceae UCG-002 (LDA score = 4.5, P adj. < 0.001), Rikenellaceae_RC9_gut_group (LDA score = 4.3, P adj. = 0.002), Butyricicoccaceae UCG-008 (LDA score = 3.9, P adj. = 0.040) and *Methanobrevibacter* (LDA score = 3.8, P adj. < 0.001) (Fig. 3A). At d34, the CO treatment was characterised by a higher abundance of *Coprococcus* (LDA score = 3.66, P adj. = 0.04) and *Oscillospira* (LDA score = 3.52, P adj. = 0.01) and the Blend1 treatment was characterised by a higher abundance of *Methanosphaera* (LDA score = 4.11, P adj. = 0.002) (Fig. 3B).

Morphometric analysis

Table 4 reports the effect of the diet on the morphometric parameters to analyse at d14 and d35 postweaning. No significant effects of the diet were observed at d14. At d35, no significant

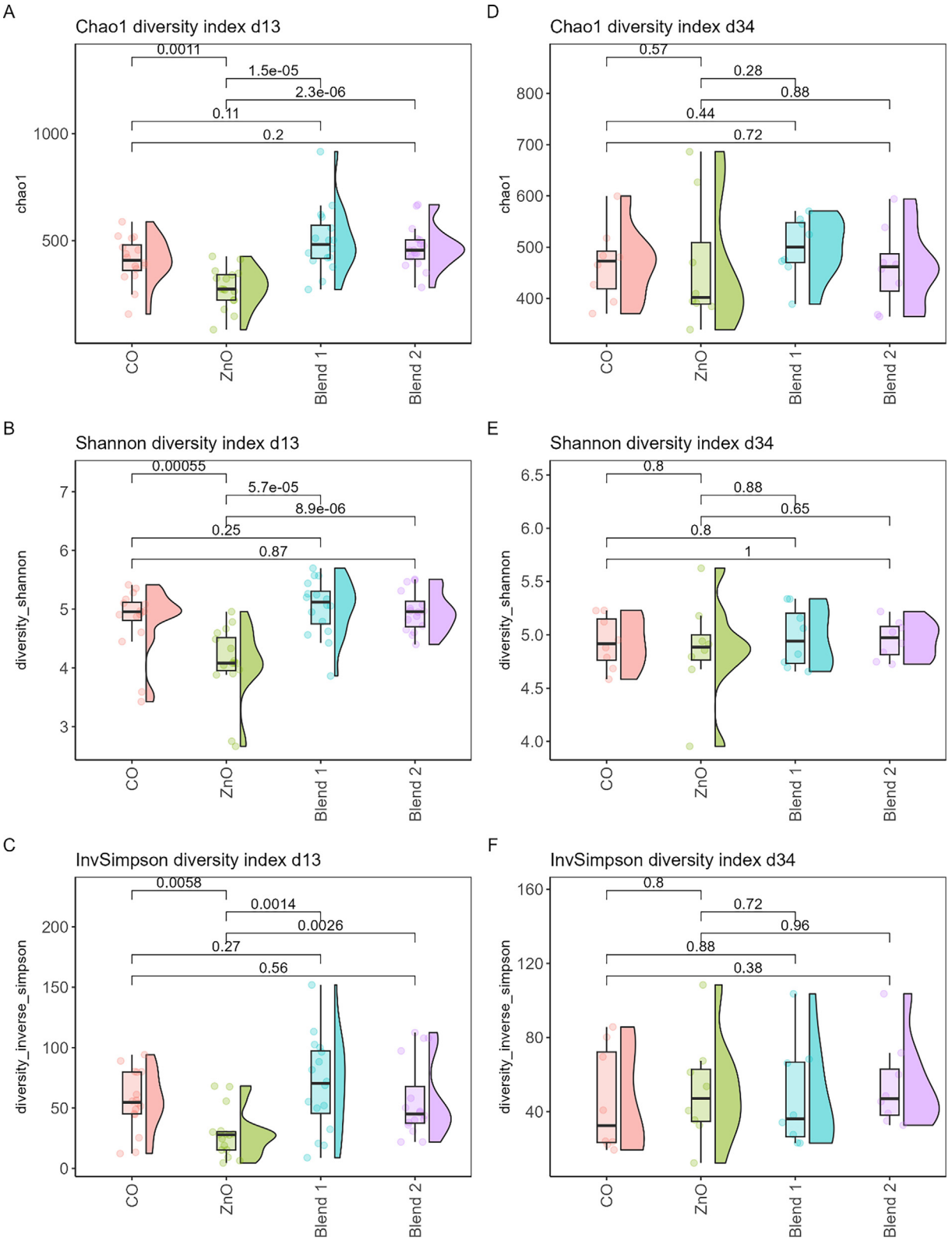

Fig. 1. Effect of supplementation with Blend1 and Blend2 on alpha diversity indices of postweaning piglets faecal samples collected at days 13 and 34. Chao1 index (A;D), Shannon index (B;E) and Inverse Simpson (InvSimpson) index (C;F). CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic).

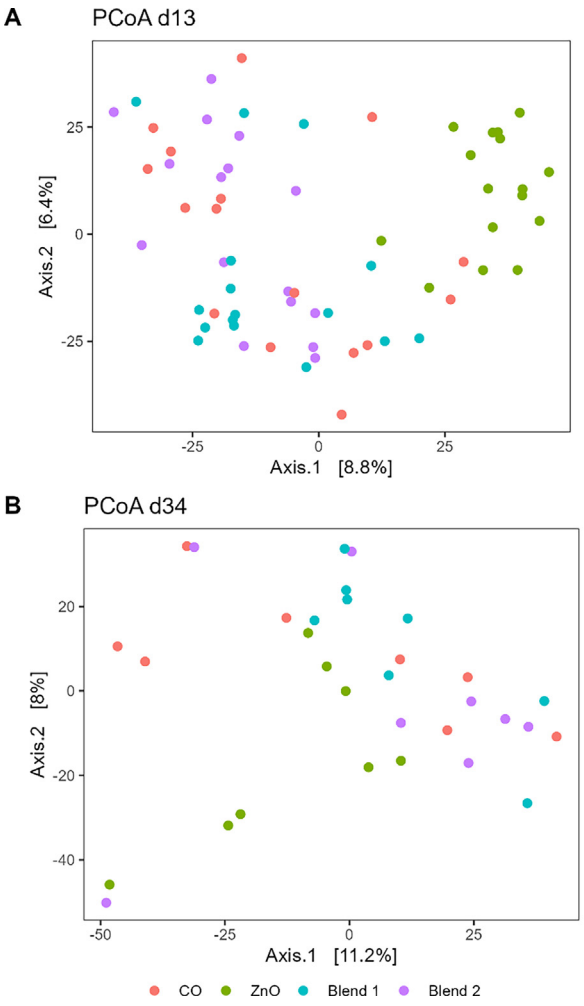

Fig. 2. Effect of supplementation with Blend1 and Blend2 on beta diversity of postweaning piglets' faecal samples collected at day 13 (A) and day 34 (B). Principal Coordinates Analysis plot (PCoA) were generated using a Euclidean distance matrix based on centred log-ratio transform transformed data. CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic).

effects of the diet were observed for the villus width, crypt depth, crypt width and absorptive mucosal surface. At d35, a significant effect was observed for the villus height (P = 0.05); the CO treatment had higher villus height (P = 0.04) than Blend2 with intermediate value for ZnO and Blend1 treatments; furthermore, a trend for a significant effect was observed for the villus height: crypt depth ratio (P = 0.08); the CO tended to have a higher villus height: crypt depth ratio than the Blend2 (P = 0.08) with intermediate value for ZnO and Blend1 treatments.

Reactive oxygen metabolites and gene expression

No significant difference was observed between treatments for the reactive oxygen metabolites at d14 (P = 0.81; CO = 24.1; ZnO = 25.7; Blend1 = 23.5; Blend2 = 24.3, data reported in Carratelli Units) and d35 (P = 0.90; CO = 26.6; ZnO = 28.9; Blend1 = 27.0; Blend2 = 28.4, data reported in Carratelli Units).

The results of the gene expression on the jejunal mucosa of piglets at d14 and d35 are shown in Supplementary Fig. S2. No effects of the diet were observed for any gene at d14 and d35 (P > 0.10).

Discussion

The use of natural and natural identical essential oil components in livestock animals has seen a recent increase in their use as a result of the need to reduce the use of antibiotics. In postweaning piglets, they would play a valid strategy in reducing the use of pharmacological doses of ZnO (Rebucci et al., 2022).

In the present study, two different blends of natural and natural identical essential oils, one of which was formulated in combination with organic acids, were evaluated in comparison with the pharmacological dose of ZnO or a normal postweaning diet on the health, growth performance and gut health of postweaning pigs. Since the two blends had a different composition, the evaluation of added value in the use of organic acids to the essential oils components was not the main objective of the present study. In general, the *in vivo* trial was carried out in animals characterised by a good state of health, as evidenced by the faecal score data which were under the threshold of diarrhoea (>3) throughout the whole trial. Furthermore, the fact that the CO treatment and the treatment fed with the pharmacological dose of ZnO did not differ in terms of ADG, FI and F:G, as observed in previous studies (Hung et al., 2020; Liu et al., 2020) supports that piglets were generally healthy and no problems were observed during the trial. Since the animals were healthy, it was also possible to highlight the different mechanism of action of these blends compared to that of ZnO at pharmacological doses, whose mechanism of action is generally mostly studied in challenge conditions. It should be noted that in this study, the pharmacological use of ZnO significantly modified the intestinal pH and microbial ecosystem of the piglets. Indeed, an increase in the cecal pH was observed in the ZnO treatment, thus it can be associated with a reduction in the production of short-chain fatty acids by intestinal bacteria (Heo et al., 2013). Furthermore, ZnO affected the overall microbial ecosystem as the beta diversity showed a greater dispersion of the microbial community and the alpha diversity indices were lower compared with the other treatments. Especially under stressful situations, including weaning, an increase in alpha diversity indices has been frequently associated with a more stable and mature microbiota due to an increase in bacterial functional redundancy (Luise et al., 2021). The use at pharmaceutical doses of ZnO had been previously associated with a reduction in sample dispersion and alpha diversity indices in weaned pigs as observed previously by Ortiz Sanjuán et al. (2022); thus, our data confirm this observation.

The supplementation of Blend1 which is composed of protected cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol) and clove essential oil (titrated in eugenol) did not significantly improve the ADG or affect the FI compared with the CO diet and the diet with the pharmacological ZnO. However, considering the F:G from d0 to d14, the Blend1 tended to have a higher value compared with the CO treatment.

Although the results on the performance showed negligible impact, the supplementation of Blend1 strongly influenced the gut ecosystem and gut health of the postweaning piglets in a different way compared with the pharmacological dose of ZnO and CO treatments. Indeed, the Blend1 led to a reduction of the haemolytic *E. coli* compared with the CO treatment, which is a typical pathogen responsible for the postweaning diarrhoea (Luppi, 2017). The reduction in the haemolytic *E. coli* has been proposed as markers for better gut health (He et al., 2022). Although the count of the haemolytic *E. coli* was low in the present study, this

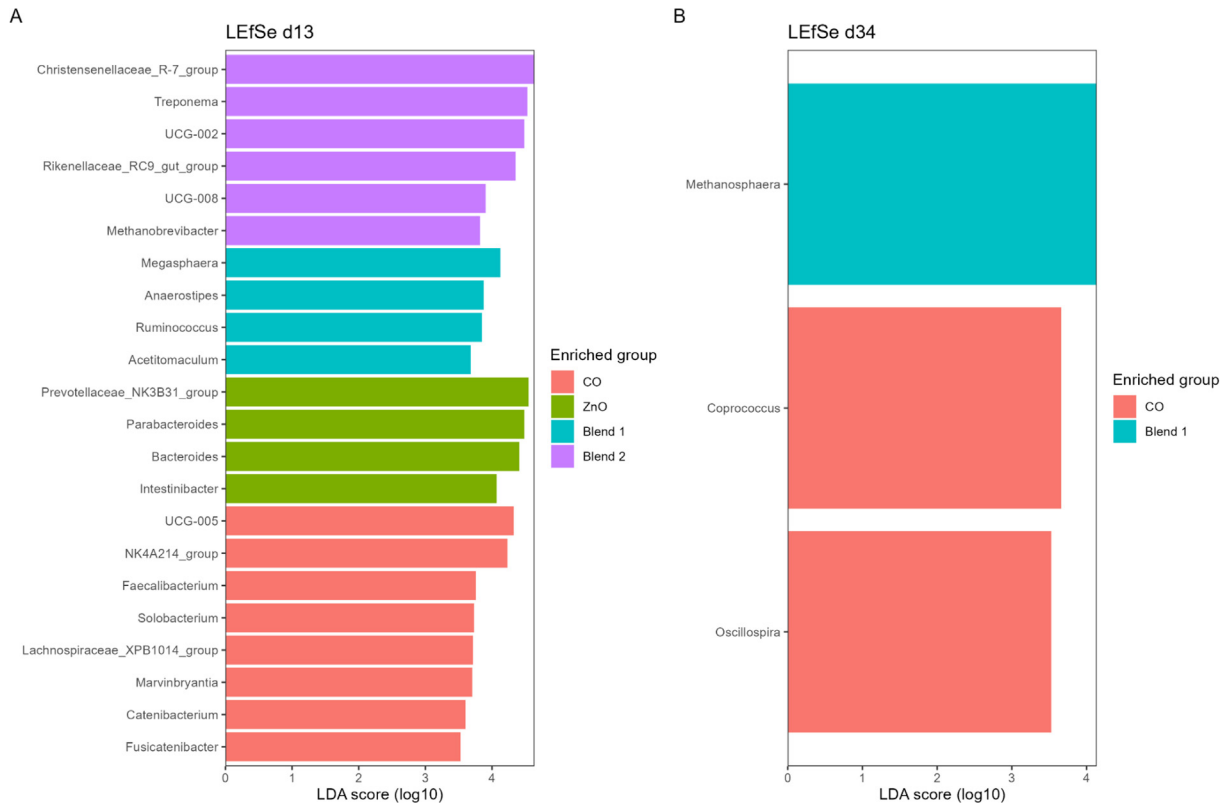

Fig. 3. Effect of supplementation with Blend1 and Blend2 on microbial biomarkers at genus level in postweaning piglets' faecal samples collected at days 13 (A) d34 (B). LEfse = Linear discriminant analysis Effect Size analysis; LDA score: Linear Discriminant Analysis score; CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic).

Table 4
Effect of supplementation with Blend1 and Blend2 on morphometric parameter in the jejunum of postweaning piglets.

Items	Diet[1]				SEM	*P*-value
	CO	ZnO	Blend1	Blend2		
d14, µm						
Villus height	289	292	273	247	25.3	0.59
Villus width	105	116	107	104	5.1	0.32
Crypt depth	226	239	210	236	22.9	0.80
Crypt width	50.1	53.1	63.9	48.1	8.0	0.52
Villus height: Crypt depth ratio	1.30	1.28	1.71	1.12	0.3	0.52
Absorptive mucosal surface	5.50	5.26	5.38	5.00	1.2	0.83
d35, µm						
Villus height	279^{A}	267^{AB}	256^{AB}	234^{B}	11.0	0.05
Villus width	91.1	93.1	95.4	91.8	5.8	0.96
Crypt depth	164	164	171	169	6.6	0.85
Crypt width	30.6	28.7	31.6	30.7	1.0	0.25
Villus height: Crypt depth ratio	1.74^{a}	1.64^{ab}	1.50^{ab}	1.39^{b}	0.1	0.08
Absorptive mucosal surface	7.42	7.24	6.62	6.25	0.4	0.18

Diet[1]: CO = basal diet without antimicrobials and pharmacological level of Zn; ZnO = CO diet supplemented with ZnO at a pharmacological dose (2 400 ppm) only from d0 to d14; Blend1 = same diet as the CO treatment but integrated with the 150 g/100 kg feed of an encapsulated mixture of cinnamaldehyde (natural identical), cassia oil (titrated in cinnamaldehyde), ajowan essential oil (titrated in thymol), essential oil of clove (titrated in eugenol); Blend2 = same diet as the CO treatment but integrated with 200 g/100 kg of feed of an encapsulated mixture of fatty acids in ester form (butyric, capric and caprylic; total 400 g/kg), eugenol (natural identical synthetic), cinnamaldehyde (natural identical synthetic), methyl salicylate (synthetic). Different capital letters indicate significant differences between treatments (*P*-value < 0.05), while lowercase letters indicate a trend of significance between treatments.

effect of Blend1 confirms the antibacterial effects of cinnamaldehyde, ajowan essential oil and clove essential oil on pathogens, as previously reported by *in vitro* studies (Chaieb et al., 2007; Goudarzi et al., 2011). Furthermore, the present results agree with the results of Samanta et al. (2021), which study a blend of *Cinnamomum zeylanicum* and *Trachyspermum copticum* essential oils (0.3 g/kg and 0.4 g/kg, respectively) increases the number of the *Lactobacillus* and reduced the number of enterobacterial in the faeces of postweaning pigs. Similarly, the supplementation of 1 000 mg eugenol/kg or 1 000 mg cinnamaldehyde/kg reduced the faecal *E. coli* concentration in weaned piglets (Yan and Kim, 2012). According to the literature, one of the main mechanisms

by which thymol, carvacrol, eugenol and cinnamaldehyde can inhibit the proliferation of the pathogens is related to their effects on the cytoplasmic membranes and energy metabolism, as they can inhibit histidine decarboxylase activity and bind proteins (Omonijo et al., 2018). In addition, in the present study, it has been observed that the supplementation of a blend of cinnamaldehyde, ajowan essential oil (*Trachyspermum ammi*) and clove essential oil can modify not only specific pathogens but also commensal bacteria, which, in turn, may have contributed in the reduction of haemolytic *E. coli*.

In fact, the Blend1 diet led to an increasing in the abundance of *Acetitomaculum*, which is an acetate-producing genus (Greening and Leedle, 1989) and *Ruminococcus* and *Megasphaera* which are both known to produce butyrate (Claesson et al., 2012; Counotte et al., 1981). In agreement with the present study, the data of da Silva et al. (2022) showed that the supplementation of phytogenic with a similar composition (essential oils: 41% garlic oil, 6% essential oil of cinnamic aldehyde, thymol, carvacrol and eugenol) of Blend1 to weaned piglets promoted the cecum abundance of *Megasphaera*. In addition, *Ruminococcus* and *Megasphaera* were reported to increase by the supplementation of essential oils (carvacrol: 62.5 mg/kg and thymol: 7.5 mg/kg) in the study of Li et al. (2018); therefore, these results confirm an effect of essential oil on these specific taxa. The study of Li et al (2018) suggested that the essential oil modified also the microbial functions and especially the carbohydrate and amino acid metabolism. The changes in intestinal environmental conditions, microbial population and their metabolism could be expected to affect the expression of the intestinal genes; however, no differences were observed in the present study. This is probably due to the general good health of the piglets included in the study.

The supplementation of Blend2, which was composed of cinnamaldehyde, eugenol, methyl salicylate and mono and diglycerides of propionic, butyric, caprylic, capric acids, lead some modification in the gut microbiota compared to the ZnO treatment. Indeed, the Blend2 had a higher alpha diversity compared with the ZnO, promoted the colonisation of specific taxa and reduced the intestinal pH compared with the ZnO; however, these changes in the gut did not lead to an effect on the performances. The Blend2 reduced the growth performance of the postweaning piglets compared to both the CO and the ZnO treatments, especially during the first fourteen days postweaning. Several studies suggested that a mixture of synthetic essential oils and organic acids may have a positive additive effect on the modulation of intestinal pathogens (Hulánková and Bořilová, 2011) and promote the growth and health of postweaning piglets (Caprarulo et al., 2022). However, other studies suggested that the positive results can vary according to the dose of integration, indeed too high doses compromised the performance in studies of Rodrigues et al. (2020) and Zhai et al. (2020).

It is also recognised that essential oils and organic acids and their salts can have strong and unpleasant smells which can reduce feed palatability and FI of pigs (Zentek et al., 2011), as observed in the present study during the first fourteen days. As an additional negative effect, the supplementation of Blend2 significantly reduced the villus height and the VH:CD ratio and the absorptive surface in the jejunum, suggesting a reduction in the functional capacity of the gut. The reduction in the functional capacity of the Blend2 could also be associated with the limited FI of this treatment of pigs; in fact, it is well known that a low FI can be responsible for villous atrophy, especially in the postweaning period (Dong and Pluske, 2007).

These negative effects of Blend2 may be due to a not optimal encapsulation of the product or to a too-high dose of the active compounds. Indeed, as suggested by Abdelli et al. (2020) and Timbermont et al. (2010), higher dietary levels of phytoextracts and medium-chain fatty acids were associated with the compromised productive performance of broilers, due to shorter villus height, deeper crypts, reduced VH:CD ratio.

Conclusion

In conclusion, including natural and natural identical essential oil mixture in a postweaning diet could be a potential strategy to maintain the gut health of postweaning piglets. However, the efficacy of these blends varies accordingly to their composition and inclusion in the diet. The dietary supplementation of Blend1 at a dose of 150 g/100 kg of feed allowed to modulate of the gut microbiome promoting the abundance of short-chain fatty acid-producing bacteria and reducing the one of haemolytic *E. coli*, therefore, it could be evaluated as a potential strategy to maintain the gut health of postweaning piglets.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.101031.

Ethics approval

The procedures complied with Italian law on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, Italy and by the Italian Ministry of Health (Project ID 1280; Code 2216A.N. NFJ). The animal trial was performed at the experimental facility of the University of Bologna.

Data and model availability statement

The raw reads obtained from the microbiota DNA are publicly available at the Sequence Read Archive (SRA) under the accession number: PRJNA940816 at: https://www.ncbi.nlm.nih.gov/bioproject/?term=(PRJNA940816)%20AND%20bioproject_sra[filter]%20NOT%20bioproject_gap[filter]. Information can be made available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

Author ORCIDs

DL: https://orcid.org/0000-0001-7864-7822.
FC: https://orcid.org/0000-0001-8558-6503.
CN: https://orcid.org/0000-0003-0895-0070.
SV: https://orcid.org/0000-0002-4251-3328.
MM: https://orcid.org/0000-0002-7384-5990.
PT: https://orcid.org/0000-0001-7019-6828.

Author contributions

PT designed the experiment. **DL**, FC, SV and CN performed the experiment and collected samples. DL, FC, MM and CN analysed the samples and data. DL and FC conceptualised the paper, compiled all of the information and prepared the manuscript. PT conceptualised the paper, provided insights into the entire manuscript and contributed to the writing.

Declaration of interest

The authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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