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Effect of an *Escherichia coli* F4/F18 bivalent oral live vaccine on gut health and performance of healthy weaned pigs

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ABSTRACT

Oral live vaccines stimulate host immunity, but they could also affect intestinal mucosa development and gut microbiota of piglets during the postweaning. The aim of this study was to determine the effect of an oral vaccine against *Escherichia coli* F4 and F18 (Coliprotec F4/F18®), on gut functionality and integrity, growth performance and health status of postweaning piglets. A total of 96 weaned piglets (23.30 ± 1.85 days of age; 7334 ± 1039 g BW) were divided into two groups (16 replicates/group; three piglets/replicate) as follows: (1) Control (**CO**), fed a standard diet (prestarter up to 14 days, then starter feed); (2) Treated (**TRT**): as CO but vaccinated with Coliprotec F4/F18® at weaning (day 0). Piglets were weighed at day 0 and weekly until day 35. Individual faecal score was recorded daily. Piglets were sacrificed at days 10 (1/3 of total) and 35 (2/3). Samples of jejunum mucosa and of cecum content were collected for morphometric, immunohistochemistry analysis and for microbiota profile analysis, respectively. Data were fitted using a linear model including treatment, class of starting BW as fixed factors and litter as random factor. From days 0 to 7, piglets from the TRT group tended to have a higher average daily gain (+22.6%, $P = 0.08$) and average daily feed intake compared to the CO group (+13.2%, $P = 0.022$). Gain to feed ratio was lower in the TRT group from days 14 to 35 (−6.6%, $P = 0.011$). From days 7 to 14, the TRT group had a higher diarrhoea index (−199%, $P < 0.001$). Crypt depth was higher in the CO group (+10.9%, $P = 0.04$) at day 10, but not at day 35. Jejunal expression of Claudin-4 (probability of having a score = 3) was higher in the TRT group at day 10 (CO = 1.50% vs TRT = 2.69%, $P < 0.0001$) and day 35 (CO = 1.29% vs TRT = 1.92%, $P = 0.012$). Oral vaccine affected beta diversity at day 10 ($P = 0.040$; $R^2 = 0.05$) and increased the abundance of specific taxa and genera in the cecum at day 10, including *Prevotella* (lg2FC = 23.2, FDR < 0.001). The results showed how an *Escherichia coli*-based vaccine supplied to weaned pigs can promote gut health by controlling symptoms of the postweaning perturbation in the first 2 weeks postweaning. In addition, the vaccine strains showed a probiotic-like effect by modulating gut microbiota favouring the establishment of beneficial bacteria, and by promoting gut barrier integrity.

Implications

Findings reported in this study evidenced how the administration of an oral vaccine against enterotoxigenic F4/F18 *Escherichia coli* strains at weaning favours feed intake, stabilises enterocyte turnover, improves intestinal barrier and the settlement of beneficial bacteria, and reduces diarrhoea in the first-phase postweaning. Thereafter, vaccination can be associated with a transient reduction of feed efficiency, but still associated with good markers of gut microbiome and intestinal barrier with no negative effect on standard blood parameters. These factors may indicate a possible improvement of piglet resilience, also based on previous results on the long-term effect of vaccination under commercial farm conditions.

Introduction

Postweaning diarrhoea (**PWD**) represents one of the major threats in weaned pigs' management, leading to a marked decrease in feed consumption and an increase in morbidity, mortality, and

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economic losses (Luppi, 2017). The F4 and F18 strains of Enterotoxigenic *Escherichia coli* (**ETEC**) are the most isolated enteric pathogens from piglets in the weaning period and the primary cause of PWD (Luppi, 2017). The ability of these strains to produce a protein appendage (F4 or F18 fimbriae), able to adhere to specific receptors in pigs' small intestine, is a prerequisite for developing their pathogenicity, which is conveyed by the production of several heat-stable and/or heat-labile toxins (Van den Broeck et al., 1999). The ETEC ability to adhere to the intestinal villi is also necessary in order to produce an intense and persistent humoral immune response by the piglet (Van den Broeck et al., 1999). Thus, strains carrying the ability to produce F4 or F18 but unable to synthesise toxins could stimulate immunoglobulin production, without inducing the disease. The use of single or bivalent vaccines for ETEC F4 / F18 has been experimentally tested with positive effects in reducing clinical signs of PWD and ETEC faecal shedding after vaccination of ETEC challenged piglets (Fairbrother et al. 2017; Nadeau et al., 2017). However, as reported in our previous study, the administration of the oral bivalent vaccine for ETEC showed that the vaccine, besides protecting the animals from the *E. coli* infection and PWD (Luise et al. 2020) and stimulating the specific immune response to ETEC strains (Fairbrother et al. 2017; Nadeau et al., 2017), can also modulate faecal microbiota. This effect may be linked to the administration of 10^8 –10^9 live *E. coli* contained in the vaccine, which can have a direct effect on the microbial community. Furthermore, it is known that the microbiota and the host are in a continuous cross-talk (Patil et al. 2020). Therefore, the administration of the ETEC bivalent vaccine based on avirulent strains of *E. coli* F4 and F18 could act as a microbial modulator resulting in a modulation of the gut mucosa state, the non-specific immune tolerance and oxidative status of piglets. Following our preliminary results (Trevisi et al. 2022), the aim of the present study was to determine the effect of an oral vaccine administration against *Escherichia coli* F4 and F18, on growth performance, health, oxidative status, intestinal integrity, and gut microbiota of piglets from weaning until 4 weeks postweaning.

Material and methods

Experimental procedures

At weaning (23.30 ± 1.85 days of age; day 0), a total of 96 piglets (Large white × Landrace × Duroc) (7 334 ± 1 039 g BW – BW0), 48 castrated males and 48 females were transported from a commercial farm to the experimental unit of the University of Bologna.

Piglets were taken from a farm where the bivalent vaccine Coliprotec F4/F18® was not adopted yet and where ETEC F4 or F18 were isolated in previous production cycles. The pigs were divided into two experimental groups as follows: (1) Control group (**CO**), assigned to a standard dietary treatment used for postweaning piglets; (2) Treated group (**TRT**): as CO but orally inoculated with 2 mL of live non-pathogenic *E. coli* O8:K87 (F4ac+) and O141:K94 (F18ac+) vaccine (Coliprotec F4/F18®, Elanco Italia, Sesto Fiorentino, Italy) on the day of weaning. A single-dose of lyophilised vaccine consists of live non-pathogenic *E. coli* O8:K87 (F4ac-positive, 1.3×10^8–9.0×10^8 CFU) and O141:K94 (F18ac positive, 2.8×10^8–3.0×10^9 CFU). After being rehydrated in water, the vaccine was orally administrated using a dosing device.

Pigs of each group were divided into 16 replicates (3 piglets/replicate) balanced for the litter of origin and initial BW. Animals were fed *ad libitum* and had continuous access to water. The replicates (box) were classified into three classes of BW0: low (L; BW0 < 6 500 g; eight replicates, four for CO and four for TRT groups), medium (M; 8 100 < BW0 > 6 500 g; 16 replicates, eight for CO and eight for TRT groups) and high (H; BW0 > 8 100 g; eight replicates, four for CO and four for TRT groups) according to their average BW at day 0. Animals were fed with two-phase diets: phase I from days 0 to 14 postweaning and phase II from days 15 to 35 postweaning. The calculated and analysed dietary contents are reported in Supplementary Table S1a and Table S1b.

Pigs treated and not with Coliprotec F4/F18® were housed separately to avoid cross-contamination. Pigs were kept in controlled environmental conditions, where temperature and humidity were recorded daily; additionally infrared lamps were used during the first 7 days postweaning.

Measurement and sample collection

Piglets were individually weighed at day 0 and weekly until the end of the trial (day 35 postweaning). Feed intake of each replicate was daily recorded. General health status and faecal consistency (based on 5 points: 1 hard faeces – 5 watery faeces) were daily monitored and recorded as described by Luise et al. (2019). Piglets were considered to have diarrhoea when the faecal score was ≥3. Days of diarrhoea in each week were then counted and expressed as a percentage on seven days (diarrhoea index).

Piglets were euthanised at two time points: day 10, one piglet/replicate (total 32 piglets) (acute postweaning phase) and day 35, the remaining pigs (endpoint). At the first time point, sacrificed piglets were selected based on the average daily gain (**ADG**) of the group, in order to respect the differences among groups in terms of the growth curve. Slaughtering was performed using 0.5 mL/kg BW intracardiac injection of Tanax (containing tetracaine hydrochloride, mebenzonium iodide and embutramide) (Intervet Productions Srl, Aprilia, Italy), after pigs sedation with 15 mg/kg BW Zoletil 100 (Virbac, Milano, Italy).

Blood samples were collected at day 10 (from pigs sacrificed and from one additional piglet/replicate), day 21 (one piglet/replicate) and day 35 (one piglet/replicate) from vena cava using a collection tube with K3 EDTA (Vacutest Kima Padova, Italy) to analyse blood cell formula and using a collection tube with clot activator (Vacutest Kima) to obtain serum. From non-sacrificed animals, blood was drawn by placing the animals in dorsal recumbent and by securing their head, hinds and forelimbs.

From slaughtered pigs, the intestine was immediately removed. The distal part of the jejunum (starting approximately from the first 75% of the small intestine length) was sampled for morphological and immunohistochemical analyses. Moreover, a sample of cecum content was collected to determine the microbial profile.

Haematological and oxidative parameters

A total of 15 haematological parameters (red blood cell count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, lymphocyte, neutrophil, eosinophil, basophil, monocyte, and platelet count) were detected by laser-impedimetric-cytometry using the automatic analyzer CELL-DYN 3700R® (Abbott Laboratories; Abbott Park, IL, USA).

Reactive oxygen metabolites (**ROMs**) as indicator of intestinal mucosa damage were quantified in duplicates in blood serum (obtained by centrifuging blood tubes at 3000 g for 10 min) colourimetrically with d-ROMs test kit (Diacron International, Grosseto, Italy) following the automatic method of Brambilla et al. (2002). Briefly, after 1:20 dilution in distilled water, the serum was kept for 5 min at 37 °C in a solution with 0.01 M acetic acid/sodium acetate buffer pH 4.8 and N,N diethyl p-phenylenediamine, and then, absorbance was registered at 520 nm.

Microbiota analysis

FastDNA SPIN Kit for Soil (DBA Italia, Segrate, Italy) was used for total bacterial DNA extraction from 64 caecal samples (16 samples per group per time point). The V3-V4 hypervariable regions of the 16S rRNA were amplified using Pro341F: 5′-TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GTCTCGTGG-GCTCGGAGATGTGTATAAGAGACAGGACTACNVGG GTATCTAATCC-3′ specific primers. Library formation and amplicon sequencing were carried out using the MiSeq® Reagent Kit V3-V4 (Illumina Netherlands, Eindhoven, Netherlands) on a MiSeq-Illumina® platform.

Bioinformatic analysis was carried out as described by Luise et al. (2020). In brief, generated sequences were analysed using the DADA2 package version 1.5.0 and workflow (Callahan et al., 2016) in R version 3.6 (http://www.R-project.org). Briefly, primers were removed, and forward reads were trimmed at 280 bp, while reverse reads were trimmed at 210 bp to remove low-quality tails. Reads containing ambiguous bases and expected error rate ≥ 2 were filtered out. Reads with identical sequences were collapsed to reduce the computational time. Amplicons were generated using the DADA2 algorithm with default parameters. The output of forward and reverse reads was merged, and reads with mismatches were removed. Amplicon sequence variants (**ASVs**) shorter than 245 were removed. Chimaeras were identified and removed by ChimeraDenovo function. Taxonomy was assigned using the Silva Database (release 138.1) (Quast et al., 2013).

Morphological and immunohistochemical analyses

Jejunum samples were fixed overnight (10% neutral buffered formalin) and embedded in paraffin. For morphometric evaluation, paraffin tissue sections were deparaffinised in xylene and stained with haematoxylin-eosin. Each sample was measured for height and width of 10 villi and depth and width of 10 crypts (randomly selected). Mucosal-to-serosal amplification ratio (M) was calculated as following: M = (villous surface + unit bottom − villous bottom)/unit bottom, where villous surface = $\pi \times$ (villous length $\times$ villous width), unit bottom = $\pi \times$ (villous width/2 + crypt width/2)2 and villous bottom = $\pi \times$ (villous width/2)2, according to Kisielinski et al. (2002).

Claudin-4 was selected as a representative "tightening" protein to be evidenced in the intestinal epithelial cells of the pigs (Pasternak et al., 2015). For the immunohistochemistry, sections were deparaffinised with xylene and rehydrated using a graduated series of ethanol. To unmask antigenic sites, slides were heated in 10 mmol/L sodium citrate buffer (pH 6.0) for two periods of 5 min each in a microwave oven at 750 W. Successively, sections were incubated for 30 min in PBS containing 10% appropriate normal serum and 1% bovine serum albumin to prevent antibodies non-specific binding. Sections were then incubated overnight at 4 °C with mouse anti-Claudin- 4 monoclonal antibody (isotype IgG1, Clone 2E2C1, cat. n° 32-9400, Invitrogen/Thermo Fisher Scientific, Inc.). After washing, sections were incubated at room temperature for 1 h with anti-rabbit biotinylated secondary antibody and, subsequently, treated with avidin-biotin kit and, as a chromogen, brown 3,3′-diaminobenzidine. Sections were then counterstained with Toluidine blue. Preparations were examined on a Nikon Eclipse Ni microscope equipped with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the Fig. panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).

As staining was not uniform and often varied between the villus sides and tip cells, a score was given for staining intensity at the villus tips and the villus sides by the same operator. Sections were scored based on the presence, distribution, and intensity of Claudin-4 immunoreactivity in 20 villi with the following score categories: 1 = light/scarce staining, 2 = moderate staining, 3 = intense staining (Fig. 1A).

Statistical analysis

All statistical analyses were carried out in R v3.6 (R core team, 2021) using "car", "lm4" and "lsmeans" packages. Data were fitted using a linear model in which treatment (CO and TRT) and class of BW0 (Low, Medium, High) were included as fixed factors and litter of origin was included as a random effect. Interaction between treatment and class of BW0 was initially included in the model but then discarded because always not significant. For BW, ADG, average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**), box was used as experimental unit. For the diarrhoea index, a Poisson generalised linear mixed model was carried out.

For Claudin-4, Fisher's exact test was used to assess if vaccination contributes to the response variable distribution.

Comparisons among treatments were tested using a posthoc test (Tukey test).

Microbial biostatistical analysis

Abundance data were normalised using centred log-ratio transformation. Alpha (Shannon, Chao1 and InvSimpson indices) and Beta diversity (calculated as Bray Curtis distance matrix), as well as the abundance of taxonomic categories, were analysed with R software v3.6, by using "phyloseq"(McMurdie and Holmes, 2013), "vegan" (Dixon, 2003) and "car" (Fox and Weisberg, 2011) packages. Alpha diversity indices were analysed with an ANOVA model, considering litter of origin, group, time of sampling (day 10 and day 35) and their interaction as fixed factors. Beta diversity was analysed with a permutational ANOVA model ("Adonis" procedure) including litter, group, time points and their interaction. Thereafter, analyses on alpha and beta diversity were carried out for each single time points separately including the litter and group as factors.

The differences in taxonomic composition at phylum, family, and genus level between CO and TRT groups were performed with the "DESeq2" package (Love et al., 2014) using negative binomial generalised linear models and the multiple testing correction of Benjamini-Hochberg (Love et al., 2014; Luise et al., 2021b). The multivariate supervised approach sparse Partial Least Squares Discriminant Analysis (**PLS-DA**), included in the R package "mixOmics" v.6.20.0 (Lê Cao et al. 2011), was carried out on microbial data aggregated at the Genus level, obtained at day 10 and day 35 to identify the discriminant taxa of each group. For each component included in the model, the optimal number of components and input variables were selected considering the averaged balanced classification error rate with centroids distance over 100 repeats of 5-fold cross-validation (Luise et al., 2021b). The stability of frequency scores of the selected ASVs was tested with 5-fold cross-validation and 100 repetitions ("perf" function), for the validation of the results. Taxa with stability >0.5 were retained and considered significant.

Results

Health and performance

A total of seven out of 96 piglets were individually treated with antibiotics (amoxicillin/clavulanic acid), for presumed infection of *Streptococcus suis,* four in CO and three in TRT, (Supplementary

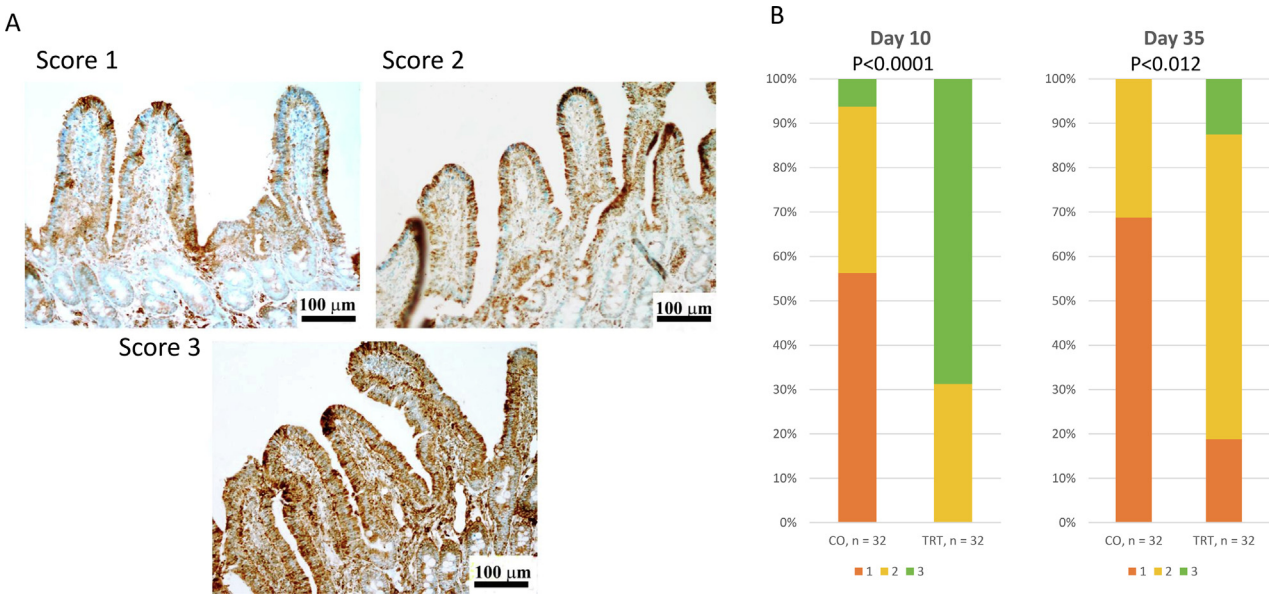


Fig. 1. (A) Representative immunohistochemistry images for Claudin-4. Score 1 = light/scarce staining: some villi and weakly marked crypts are represented; Score 2 = moderate staining: the immunoreactivity for Claudin-4 involves about 50% of the epithelial cells (enterocytes) of the villi; Score 3 = intense staining: the immunoreactivity appears intense and uniformly distributed on the surface of the villi. B) Relative frequencies of the jejunal immunohistochemistry scores for Claudin-4 (1 = light/scarce staining, 2 = moderate staining, 3 = intense staining) of postweaning piglets orally vaccinated with *E. coli* F4/F18 bivalent vaccine at days 10 and 35 postweaning. Abbreviations: CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning.

Table S2), these were not selected for microbiota, histomorphology, immunohistochemistry, oxidative stress analysis. Two piglets out of 64 (at d30 and d34, respectively) were excluded from the trial due to severe impairment of the health conditions. Both animals were in the CO group.

Fig. 2 reports the mean faecal score and diarrhoea index for CO and TRT groups during the trial. Overall, it can be observed that piglets were healthy, and the average faecal score was below 3.5 (cut-off value of diarrhoea). Nevertheless, the average score from days 0 to 7 (+1.95%, $P = 0.028$) and from days 7 to 14 (+4.41%, $P = 0.008$) was higher in CO than in TRT and tended to be higher in the CO compared with the TRT from days 0 to 35 (+2.79%, $P = 0.085$) (Fig. 2A). During the period between days 7 and d14, the TRT group had also a lower diarrhoea compared with the CO group (−199%, $P < 0.001$) (Figure2B).

Results on BW, ADFI, ADG, and G:F are reported in Table 1. The ADFI between days 0 to 7 was higher in the TRT group compared with the CO group (+13.2%, $P = 0.022$), while no differences were observed for the other periods. The ADG from days 0 to 7 tended to be higher in the TRT group compared with the CO group (+22.6%, $P = 0.072$). The ADGs calculated for the other periods were never affected by the treatment. The G:F ratio was not affected by the treatment between days 0 and 7, 7 and 14, and 0 and14, while it was lower in the TRT group compared with the CO group for the second feeding phase (−6.6%, $P = 0.011$) and tended to be lower between days 0 to 35 (−4.5%, $P = 0.069$).

Haematological and oxidative parameters

The effect of piglet vaccination on the haematological and oxidative parameters at days 10, 21 and 35 is reported in Table 2. At days 10 and 21, the vaccination did not influence the haematological parameters except for eosinophil % at day 10 for which a trend for a higher percentage was observed for the TRT group compared with the CO group (P = 0.068). At day 35, the TRT group had higher haemoglobin ($P = 0.024$), haematocrit % ($P = 0.013$), mean corpuscular volume ($P = 0.020$), number and percentage of basophil ($P = 0.019$ and $P = 0.015$, respectively) compared with the CO group. No difference in the other haematological parameters was observed.

Intestinal microbiota

A total of 1 375 926 reads at day 10 (average 44,384 sequences per sample) and a total of 2 122 636 reads at day 35 (average 66 332 sequences per sample) were obtained after sequencing. Sequences were attributed to a total of 3 651 ASVs distributed among samples. The relative rarefaction curves (Supplementary Fig. S1) showed a plateau trend for all the samples suggesting that the sequencing depth was sufficient to describe the variability within the analysed microbial communities.

The taxonomic assignment allowed to obtain 19 phyla, 71 families and 175 genera. The most abundant phylum was Firmicutes (72%) followed by Bacteroidota (23%) and Spirochaetota (1.8%). The most abundant families were Lactobacillaceae (26%), Lachnospiraceae (20%) and Prevotellaceae (19%). The most abundant genera were *Lactobacillus* (26%), *Prevotella* (10%), *Prevotella*_NK3B31 group (5%) and *Blautia* (4%).

Fig. 3 reports the microbial diversity indices and statistical effect of time and group. The alpha diversity indices increased with time ($P < 0.05$) and were also affected by the litter of origin of pigs (data not in figure, $P < 0.05$). Chao1 and Shannon indices were not influenced by treatment at any time point. The InvSimpson index was influenced by treatment at day 35 when the TRT group showed a higher value compared with the CO group (CO = 37.5, TRT = 59.1; $P = 0.04$).

For the beta diversity, the Adonis test showed that microbial composition of the samples was influenced by time ($P = 0.001$, $R^2 = 0.06$) and litter ($P = 0.002$, $R^2 = 0.15$) and not by treatment. The microbial data were then analysed separately, at day 10 and at day 35, to deeply investigate the effect of treatment. Treatment affected beta diversity at day 10 ($P = 0.040$; $R^2 = 0.05$), while it did not at day 35 ($P > 0.05$).

The differences in the taxa composition were tested using the "DESeq2" package; significant differences in taxa abundance were observed only at ASV levels and are reported in Table 3. At day 10,

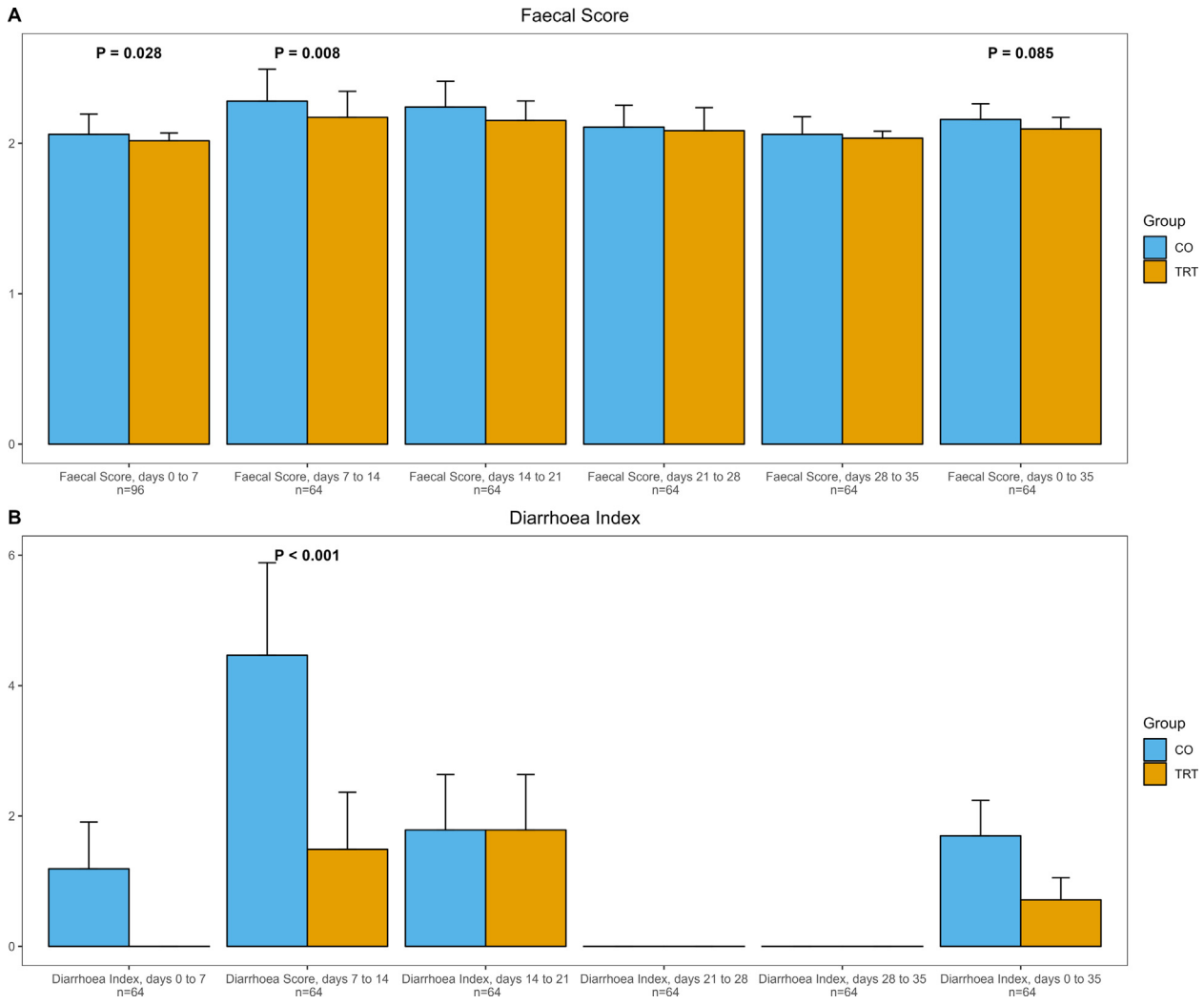


Fig. 2. Bar plots showing the effect of *E. coli* F4/F18 bivalent vaccine administration on the average faecal score (A) and diarrhoea index (B) of postweaning piglets. Abbreviations: CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning.

the CO group had a higher relative abundance of ASVs belonging to Burkholderiales Class (ASV302; *P*.Adj = 4.57E−15), *Megasphaera* (ASV152; *P*.Adj = 1.04E−13), *Phascolarctobacterium* (ASV584; *P*.Adj = 1.38E−12) and *Blautia* (ASV96 *P*.Adj = 4.00E−12); the TRT group had a higher relative abundance of the ASVs belonging to *Agathobacter* (ASV96; *P*.Adj = 4.57E−15), *Prevotella* (ASV281; *P*.Adj = 6.59E−15), and *Escherichia-Shigella* (ASV248; *P*.Adj = 1.38E−12). At day 35, the CO group had a higher relative abundance of the ASVs belonging to *Roseburia* (ASV404 and ASV422; *P*.Adj = 4.90E−27), Prevotellaceae (ASV859, *P*.Adj = 8.31E−19; ASV828, *P*.Adj = 8.31E−19; ASV122, *P*.Adj = 9.39E−14; ASV261, *P*.Adj = 0.006; ASV559, *P*.Adj = 0.05) and to the class Gastranaerophilales (ASV413; *P*.Adj = 4.14E−16); the TRT group had a higher relative abundance of the ASVs belonging to *Solobacterium* (ASV306; *P*.Adj = 4.58E−15), *Agathobacter* (ASV716; *P*.Adj = 1.03E−11), *Faecalibacterium* (ASV125; *P*.Adj = 0.01) and to the families [*Eubacterium*] *coprostanoligenes* group (ASV466, *P*.Adj = 3.85E−20 and ASV716, *P*.Adj = 1.03E−11) and Prevotellaceae UCG-001 (ASV282; *P*.Adj = 0.0003).

To identify the discriminant taxa belonging to the specific groups, the multivariate supervised approach PLS-DA was separately performed on the data for the two timepoints at the genus level (Fig. 4). At day 10, the CO group was discriminated by bacteria belonging to the genera *Selenomonas* and *Mogibacterium* and the TRT group was discriminated by *[Eubacterium] nodatum group, Prevotellaceae* UCG-004, *Escherichia-Shigella* and *Colidextribacter*. At day 35, the CO group was discriminated by *Rikenellaceae RC9* gut group, *Lachnospiraceae* XPB1014 group, *Monoglobus,* to *Family XIII UCG-001, [Eubacterium] nodatum group* and *Sutterella* and the TRT group was discriminated by *[Eubacterium] ruminantium group, Acetitomaculum, Alloprevotella, [Bacteroides] pectinophilus group, CAG-56, Mitsuokella* and *Subdoligranulum.*

Intestinal morphology and tight junction

Effects of vaccination on the morphological and immunohistochemistry parameters in the jejunum of piglets at days 10 and 35 are reported in Table 4**.** No significant differences were observed between the experimental groups for villus height, villus width, crypt width, villus to crypt ratio and M value neither at day 10 nor at day 35. The crypt depth was higher in the CO group compared with the TRT group (+10.9%, $P = 0.04$) at day 10 while it was not different at day 35.

Regarding the Claudin-4, the TRT group had a higher probability of having a higher Claudin-4 score compared with the CO group both at day 10 (CO = 1.50% vs TRT = 2.69%, $P < 0.0001$) and day 35 (CO = 1.29% vs TRT = 1.92%, $P = 0.012$) (Fig. 1B).

Table 1
Effect of *E. coli* F4/F18 bivalent vaccine administration on growth performance of postweaning piglets.

Items	Mean[1]		SEM	*P*-value	
	CO	TRT		Treatment	BW class
Live weight (g)					
Day 0	7 352	7 332	86	0.841	<0.0001
Day 14	10 557	10 793	455	0.359	<0.0001
Day 35	23 032	22 855	784	0.767	0.0002
Average daily feed intake (g/day)					
Days 0–7	136	154	5.79	0.022	0.328
Days 7–14	461	482	24.00	0.506	0.033
Days 0–14	298	318	13.70	0.271	0.039
Days 14–35	909	936	28.90	0.491	0.031
Days 0–35	664	689	23.30	0.397	0.020
Average daily gain (g/day)					
Days 0–7	84	103	6.85	0.072	0.706
Days 7–14	373	399	22.00	0.399	0.058
Days 0–14	229	250	14.30	0.291	0.133
Days 14–35	588	570	19.50	0.508	0.233
Days 0–35	444	442	15.40	0.927	0.179
Gain to feed					
Days 0–7	0.61	0.67	0.03	0.189	0.033
Days 7–14	0.81	0.82	0.02	0.542	0.014
Days 0–14	0.76	0.79	0.02	0.417	0.205
Days 14–35	0.65	0.61	0.01	0.011	0.161
Days 0–35	0.67	0.64	0.02	0.069	0.145

Abbreviations: CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning; d = day.
[1] 32 replicates (three piglets/replicate)

Table 2
Effect of *E. coli* F4/F18 bivalent vaccine administration piglet haematological blood parameters at days 10, 21 and 35 postweaning.

Item	Day 10					Day 21					Day 35				
	Mean[2]		SEM	*P*-value		Mean[3]		SEM	*P*-value		Mean[4]		SEM	*P*-value	
	CO	TRT		Treatment	BW class	CO	TRT		Treatment	BW class	CO	TRT		Treatment	BW class
RBC (10^6/uL)	6.36	6.29	0.12	0.543	0.519	6.60	6.63	0.17	0.894	0.023	7.14	7.31	0.17	0.284	0.039
HGB (g/dL)	12.00	12.10	0.28	0.519	0.442	11.50	11.60	0.33	0.700	0.076	12.00	12.50	0.22	0.024	0.017
HCT (%)	40.70	40.00	0.76	0.385	0.554	38.20	38.10	1.11	0.939	0.130	38.60	40.60	0.69	0.013	0.114
MCV (fl)	64.10	63.60	0.74	0.550	0.062	57.80	57.60	0.89	0.793	0.440	54.00	55.60	0.72	0.020	0.626
MCH (pg)	18.90	19.00	0.28	0.740	0.171	17.60	17.70	0.32	0.728	0.543	16.80	17.20	0.27	0.139	0.919
MCHC (g/dL)	29.10	29.80	0.30	0.106	0.110	30.20	30.60	0.26	0.221	0.318	31.30	30.90	0.23	0.159	0.763
RDW (%)	22.10	22.30	0.38	0.690	0.521	20.30	19.70	0.32	0.124	0.205	18.60	18.60	0.29	0.864	0.963
PLT (10^3/µL)	580.0	574.0	34.60	0.881	0.549	622.00	619.00	51.20	0.954	0.301	527.0	562.0	32.30	0.185	0.530
WBC (10^3/µL)	19.10	20.00	1.10	0.482	0.059	16.80	17.80	1.95	0.588	0.900	17.90	19.40	1.56	0.390	0.512
LYMPHO (K/µL)	9.07	9.46	0.67	0.481	0.008	10.00	10.80	1.44	0.537	0.900	12.50	14.30	1.21	0.241	0.696
MONO (K/µL)	0.47	0.54	0.04	0.215	0.276	0.34	0.37	0.04	0.546	0.279	0.40	0.49	0.07	0.337	0.226
NEUTRO (K/µL)	9.22	9.40	0.82	0.869	0.212	5.92	6.27	0.84	0.690	0.553	6.75	4.49	1.35	0.131	0.286
EOSI (K/µL)	0.25	0.30	0.04	0.353	0.910	0.26	0.23	0.03	0.378	0.004	0.20	0.22	0.03	0.441	0.018
BASO (K/µL)	0.14	0.20	0.03	0.118	0.894	0.15	0.16	0.02	0.672	0.252	0.13	0.21	0.03	0.019	0.271
LYMPHO (%)	49.10	49.10	2.24	0.994	0.259	59.20	60.70	3.38	0.631	0.431	70.40	73.30	1.84	0.199	0.779
MONO (%)	2.49	2.76	0.18	0.260	0.709	2.06	2.18	0.24	0.708	0.399	2.23	2.49	0.30	0.437	0.188
NEUTRO (%)	46.50	45.60	2.34	0.729	0.318	36.40	34.70	3.46	0.587	0.453	25.70	22.10	1.99	0.135	0.906
EOSI (%)	1.13	1.53	0.17	0.068	0.588	1.54	1.37	0.16	0.342	0.000	1.12	1.16	0.16	0.818	0.086
BASO (%)	0.75	0.96	0.12	0.138	0.969	0.87	0.93	0.13	0.755	0.119	0.74	1.03	0.11	0.015	0.199
ROM ($mmolH_2O_2$/L)	24.10	21.80	1.95	0.282	0.4289	58.20	171.10	97.80	0.347	0.202	21.00	24.20	1.49	0.087	0.126

Abbreviations: CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning; RBC = Red blood cell count, HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; RDW = Red Cell Distribution Width; WBC = White blood cell count; LYMPHO = Lymphocyte; NEUTRO = Neutrophil; EOSI = Eosinophil; BASO = Basophil; MONO = Monocyte; PLT = platelet count,; ROM = reactive oxygen metabolites.
[2] A total of 64 samples were included in the analysis (CO = 32, TRT = 32).
[3] A total of 32 samples were included in the analysis (CO = 16, TRT = 16).
[4] A total of 32 samples were included in the analysis (CO = 16, TRT = 16).

Discussion

To our knowledge, no previous study disentangled the effect of a live vaccine against ETEC infection on the gut health of healthy weaned pigs. Specifically, the tested product based on two *E. coli* strains that express F4 and F18 fimbriae can interact with the host by exerting a competitive exclusion against ETEC as well as activating the immune system. Overall, mortality, exclusion of pigs and diarrhoeal indices were low during the trial, suggesting that the health status of the animals was good. Although animals were

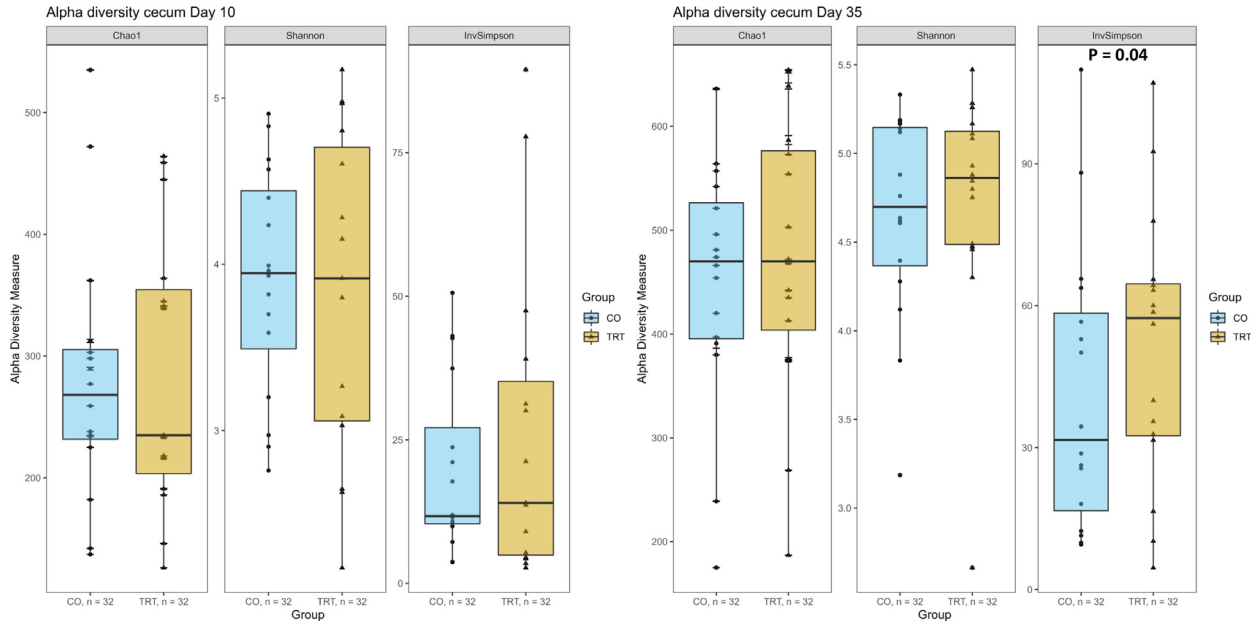


Fig. 3. Box plot of alpha diversity indices in the caecum of postweaning piglets according to their age and group at days 10 and 35 postweaning. Abbreviations: CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning.

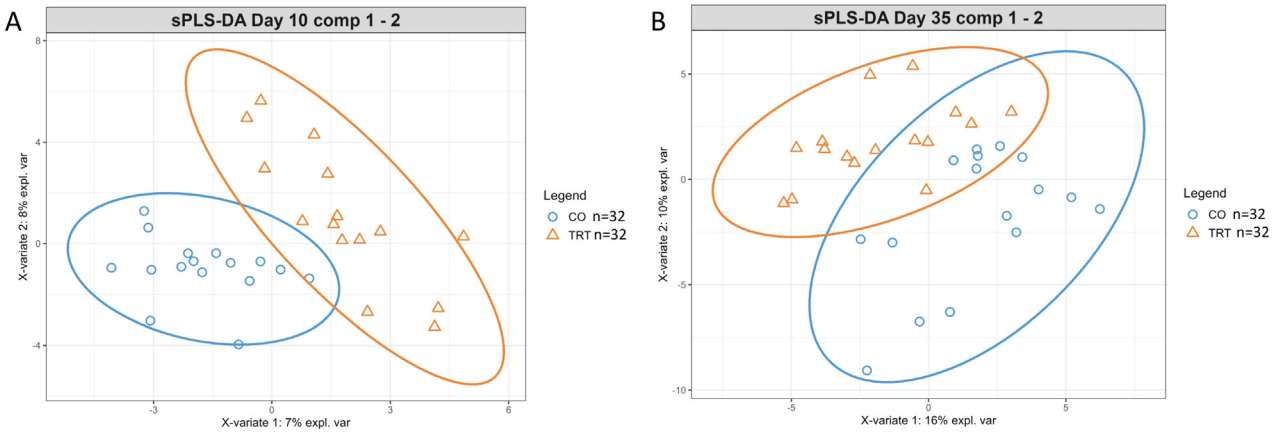
Table 3
Effect of *E. coli* F4/F18 bivalent vaccine administration on piglet caecal microbial abundance at ASVs level at days 10 and 35 postweaning.

ASVs	Taxa	log2FC	lfcSE	*P*-values	FDR
Day 10					
ASV302	Class Burkholderiales	−24.272	2.7989	4.25E−18	4.57E−15
ASV152	Megasphaera	−23.927	2.9352	3.58E−16	1.04E−13
ASV584	Phascolarctobacterium	−22.905	2.9358	6.10E−15	1.38E−12
ASV96	Blautia	−22.389	2.9354	2.40E−14	4.00E−12
ASV464	Agathobacter	23.719	2.7574	7.84E−18	4.57E−15
ASV281	Prevotella	23.291	2.7359	1.70E−17	6.59E−15
ASV248	Escherichia-Shigella	22.824	2.9326	7.08E−15	1.38E−12
Day 35					
ASV404	Roseburia	−24.951	2.19	4.42E−30	4.90E−27
ASV422	Roseburia	−24.885	2.19	5.62E−30	4.90E−27
ASV859	Prevotellaceae UCG-003	−23.74	2.50	1.91E−21	8.31E−19
ASV828	Prevotella	−23.602	2.49	2.56E−21	8.93E−19
ASV413	Class Gastranaerophilales	−23.81	2.71	1.42E−18	4.14E−16
ASV122	Prevotellaceae NK3B31 group	−23.806	2.93	4.31E−16	9.39E−14
ASV261	Prevotellaceae UCG-003	−9.2946	2.28	4.51E−05	0.006
ASV559	Prevotella	−6.8462	1.95	0.0004	0.055
ASV466	[Eubacterium] coprostanoligenes group	23.133	2.35	6.62E−23	3.85E−20
ASV306	Solobacterium	23.19	2.73	1.84E−17	4.58E−15
ASV716	[Eubacterium] coprostanoligenes group	21.718	2.89	5.30E−14	1.03E−11
ASV225	Agathobacter	21.785	2.93	1.08E−13	1.88E−11
ASV282	Prevotellaceae UCG-001	10.882	2.29	1.93E−06	0.0003
ASV125	Faecalibacterium	8.681	2.23	9.56E−05	0.012

Abbreviations: ASVs = Amplicon sequence variants; CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning; log2FC = log2fold-change, lfcSE = log fold change standard error, FDR = *P*-values adjusted for multiple comparison with false discovery rate.

healthy, the vaccine administration significantly contributed to control the symptoms of the postweaning diarrhoea syndrome. In fact, according to previous studies on ETEC-stimulated pigs (Nadeau et al., 2017), piglets of the vaccine group had a lower faecal consistency and a lower diarrhoea index compared with the control group during the first two weeks postweaning. Furthermore, the vaccinated group tended to have a higher feed intake and improved ADG during the first-week postweaning which represents the most challenging phase for weaned piglets. This latter result has not been previously reported in *in vivo* performance trials with the tested vaccine conducted in controlled or commercial conditions (Nadeau et al., 2017; Luise et al., 2020). Furthermore, in the present study, no interaction was observed between vaccine and class of BW indicating that the vaccine effects on the faecal score and growth performance were stable also for lighter pigs.

Values of erythrocyte parameters fall within the range commonly observed for weaned piglets (Perri et al., 2017). The higher haemoglobin and haematocrit values at day 35 in vaccinated pigs indicate a good oxygenation status and can be predictive of further better performance compared with control pigs (Bhattarai and Nielsen, 2015). Furthermore, leukocyte counts were constant all along the trial suggesting that no relevant general factor was perturbing vaccinated pigs during the induced immune stimulation, compared with control pigs.



C

Group	Value.var	Freq	PC	Taxa
CO	-0.29	0.60	2	Selenomona
CO	-0.09	0.54	2	Mogibacterium
TRT	0.53	0.70	2	[Eubacterium] nodatum group
TRT	0.33	0.56	2	Prevotellaceae UCG-004
TRT	0.28	0.54	2	Escherichia-Shigella
TRT	0.91	0.77	1	Colidextribacter

D

Group	Value.var	Freq	PC	Taxa
CO	0.13	0.54	1	Rikenellaceae RC9 gut group
CO	0.17	0.61	1	Lachnospiraceae XPB1014 group
CO	0.31	0.82	1	Monoglobus
CO	0.45	0.96	1	Family XIII UCG-001
CO	0.10	0.51	1	[Eubacterium] nodatum group
CO	0.28	0.81	1	Sutterella
TRT	-0.23	0.70	1	[Eubacterium] Ruminantium group
TRT	-0.19	0.65	1	Acetitomaculum
TRT	-0.16	0.61	1	Alloprevotella
TRT	-0.13	0.57	1	[Bacteroides] pectinophilus group
TRT	-0.52	0.97	1	CAG-56
TRT	-0.16	0.61	1	Mitsuokella
TRT	-0.35	0.83	1	Subdoligranulum

Fig. 4. Results of the PLS-DA analysis on faecal microbiota of piglets at days 10 and 35 postweaning. (A, C) Individual score plot of the samples along the first two components at days 10 and 35 postweaning. (B, D) Table reporting the most discriminant genera per group at days 10 and 35 postweaning; Value.var expresses the variance explained by the single genera; Freq express the frequencies by which the genera were chosen among the 100 repetitions of the cross-validation; PC stands for the principal component that discriminates the genera; Group expresses the group for which the genera were discriminant: Abbreviations: PLS-DA = Partial Least Squares Discriminant Analysis; CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning.

Table 4
Effect of *E. coli* F4/F18 bivalent vaccine administration on jejunum morphological parameters of piglets at days 10 and 35 postweaning.

Item	Day 10[1]				Day 35[2]			
	Mean		SEM	*P*-value	Mean		SEM	*P*-value
	CO	TRT		Treatment	CO	TRT		Treatment
Villus height, µm	251	232	13.20	0.265	323	335	10.90	0.370
Villus width, µm	67.30	65.60	3.79	0.723	81.90	85.70	2.83	0.323
Crypt width, µm	32.80	33.30	1.77	0.814	37.30	37.60	0.88	0.802
Crypt depth, µm	156	139	7.02	0.040	197	202	7.84	0.564
M mean[3]	7.38	6.82	0.32	0.176	8.02	8.12	0.24	0.736
Villus/crypts, µm	1.64	1.63	0.10	0.980	1.66	1.68	0.09	0.863

Abbreviations: M = Absorptive mucosal surface, M index; CO = control group; TRT = Group orally vaccinated with *E. coli* F4/F18 bivalent vaccine at weaning.
[1] A total of 32 samples were included in the analysis (CO = 16, TRT = 16).
[2] A total of 32 samples were included in the analysis (CO = 16, TRT = 16).
[3] Calculated as described by Kisielinski et al. (2002).

Vaccine ability to control postweaning diarrhoea syndrome symptoms is also evidenced by the better stabilisation of intestinal barrier function and of small intestinal mucosal turnover as suggested by the lower crypt depth and higher expression of Claudin-4 in the jejunum of vaccinated piglets. Indeed, it is generally known that the small intestine of newly weaned piglets encounters a reduction in villous height and an increase in crypt depth that can lead to a reduction in the specific activity of disaccharidase enzymes and therefore a reduced capacity to digest and absorb nutrients (Pluske et al., 1996; Hampson 1986). The increase in crypt depth is associated with an increase in crypt-cell proliferation rate and overall stimulation of cell turnover in the small intestine following an inflammation, indicating a high number of immature cells that move along the crypt-villus axis. This sign of postinflammatory recovery observed in the control group could be attributed to the direct effect of pathogens on the mucosa (Vijtiuk et al., 1995) and/or to more systemic inflammation and/or to the lower feed intake (Pluske et al., 1996) observed in this group; however, further analyses are needed to elucidate this aspect. To the authors' knowledge, no previous studies investigated

the effect of live ETEC bivalent vaccine on the villus and crypt parameters. Likewise, no previous studies investigated the effect of this vaccine on the tight junction expression. In the present study, Claudin-4 protein expression was investigated as a tight junction marker. Claudins are known to have a regulatory capacity on the pore pathway that allows small, uncharged solutes and specific ions to pass between intestinal epithelial cells (Anderson and Van Itallie, 2009), and among claudins, the Claudin-4 is the most expressed in the small intestine (Lu et al., 2013; Markov et al., 2010; Trevisi et al., 2018). For its role in the small intestine, Claudin-4 can be considered a good marker for barrier function and mucosa integrity. The higher score observed in the vaccine group suggested a positive influence of the vaccine in maintaining the barrier function and mucosal integrity in the jejunum of piglets. Indeed, the expression and score of Claudin-4 are reported to decrease with inflammation due to *E. coli* lipopolysaccharide (Ciro Galeano et al., 2014) and Porcine epidemic diarrhoea virus challenge (Curry et al., 2017).

Furthermore, as suggested by our previous study (Luise et al., 2020), it is also possible that the live ETEC bivalent vaccine had a direct modulatory effect on the gut microbiome of the piglets, including commensal bacteria. Indeed, in the present study, vaccination was not able to influence alpha diversity but modulated beta diversity and abundance of specific taxa and genera in the cecum at day 10. At day 10 postweaning, the vaccine group was discriminated by a higher abundance of *Prevotella, Eubacterium nodatum* group and *Escherichia-Shigella*. The increase of *Prevotella* agrees with our previous study (Luise et al; 2020); thus, it confirms the capacity of the vaccine to favour this genus, that is generally associated with a better maturation of the gut and with faster-growing and robust piglets (Luise et al., 2021a). The same was for the abundance of *Faecalibacterium* (Luise et al., 2020). Both these genera are frequently associated with intestinal production of short-chain fatty acids (Yu et al., 2019). Likewise, the bacteria belonging to the *Eubacterium nodatum* group are also recognised as short-chain fatty acid producers and to be higher in normal compared to low BW piglets (Li et al., 2019). In the present trial, it is possible that the production of these bacterial metabolites could have granted an optimal growth for the enterocytes (Wong et al., 2006) and a metabolic regulation that favoured the local barrier, as evidenced by the higher expression of Claudin-4. However, this modulation in the cecum microbiota was not able to determine valuable changes in the performance of piglets but it can be considered a prerequisite for the gut health and consequently a most resilient and robust pig in case of future intestinal challenges.

The increase in the *Escherichia-Shigella* in the vaccine group can be probably related to the fact that this group includes the strain used for the vaccine; therefore, this result suggests the potential colonisation of the vaccine strain in the intestinal gut of the piglets. This hypothesis should be confirmed in further studies by the quantification of the eventual specific DNA of the two vaccine strains, present in the gut content of pigs.

During the recovery phase (after the first two weeks postweaning), the present study showed that the unvaccinated piglets used fed more efficiently than piglets in the vaccine group. Our previous study suggested a potential long-term positive effect of the vaccination with ETEC bivalent vaccine on the growth performance of pigs (at d72 postweaning). However, in the present study, the *in vivo* trial was limited to 35 days. Thus, a transient effect of the vaccination cannot be excluded two weeks after the administration, which is determined by the necessary energetic need to upgrade immune activation. Particularly, the production of immunoglobulins against ETEC F18 takes more time to develop (Nadeau et al., 2017), compared with that against ETEC F4. Furthermore, the production of immunoglobulins against ETEC F18 was associated with a reduction in postvaccination growth, which was not observed after the ETEC F4 postvaccination period (Nadeau et al., 2017). Thus, it is possible that mild differences in terms of growth response or feed efficiency can be due to the balance of immune activation postvaccination against the two ETEC strains or depending on the previous immune history of the pigs. It is also worth considering that at 35 days, the InvSimpson index was higher in the caecal microbiota of vaccinated pigs, compared to the control. Compared to the other indexes, InvSimpson is less sensible to rare species, while it is mostly influenced by the most dominant species (Mouillot and Lepretre, 1999). This again is an indicator of a better ability to powerfully react in case of incoming potential destabilisers of the microbiota balance. This could fit with the previous observation of a positive long-term effect of this ETEC bivalent vaccine in commercial conditions (Luise et al., 2020).

Conclusion

The results showed that an oral live non-pathogenic *E. coli*-based vaccine supplied to weaned pigs can promote gut health by controlling symptoms of the postweaning perturbation in the first 2 weeks postweaning. In addition, the two vaccine strains showed a probiotic-like effect by modulating gut microbiota and favouring the establishment of beneficial bacteria, and by promoting gut barrier integrity. The specific immune activation induced by the two *E. coli* bacterial strains could explain the slight decrease in feed efficiency observed in vaccinated pigs during the second period.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100654.

Ethics approval

The procedures complied with Italian law pertaining to 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 (Prot. N. 33/2021-PR).

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: PRJNA788202.

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Author contributions

PT, MV, PB designed the experiment. **FC, FP, LA, SV and CN** performed the experiment and collected samples. **DL, FC, PC, MM and CN** analysed the samples and data. **DL and PB** 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. All authors read and approved the final manuscript. MV is employed by Elanco

which partially supported the experimental costs and provided the oral vaccine.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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