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Application of High Energy and Protein Diets in Combination with a Live Avirulent *Escherichia coli* F4 Vaccine Against Post-Weaning Diarrhea

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ABSTRACT

Introduction: Post-weaning diarrhea (PWD) in pigs is a worldwide economically important disease, which is frequently controlled using antibiotics. However, emergence of antimicrobial resistance in *Escherichia coli* strains urges the need for alternative control measures, such as adapted feeding strategies, pre- and probiotics, organic acids, MCFAs or immunization. **Methods:** Different alternative control strategies such as active immunization of piglets against PWD with an *E. coli* F4 vaccine (Coliprotec® F4; Elanco) combined with high energy and protein diets, addition of nutraceuticals (medium chain fatty acids (MCFAs), organic acids and additional fibers) or supplementation of ZnO were evaluated for their efficacy against PWD due to F4 enterotoxigenic *E. coli* (F4-ETEC) under field conditions. **Results:** ZnO-supplemented piglets had a lower overall end weight and lower average daily weight gain, as compared to *E. coli* vaccinated piglets. The *E. coli* vaccinated group with normal energy and protein diet had the lowest clinical scores, whereas piglets fed a ZnO-supplemented diet had intermediate fecal clinical scores. All *E. coli* vaccinated groups had a low number of antibiotic treatments. In the nutraceutical group, clinical scores were much higher, indicating more severe clinical diarrhea, which needed additional antibiotic intervention. **Conclusions:** The present study demonstrated the efficacy of an oral live non-pathogenic *E. coli* F4 vaccine for active immunization of piglets against PWD due to F4-ETEC under field conditions. Different feeding strategies had no significant effect on the clinical outcome and performance parameters of *E. coli* vaccinated piglets.

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INTRODUCTION

Post-weaning diarrhea (PWD) in pigs is a worldwide economically important disease [1], characterized by increased mortality, weight loss, retarded growth, increased treatment costs, higher use of antibiotics and batch-to-batch variation [2-8]. Enterotoxigenic *Escherichia coli* (ETEC) is regarded as the most important cause of PWD. The ETEC pathotype is typically characterized by the presence of fimbrial adhesins, which mediate attachment to porcine intestinal enterocytes, and enterotoxins, which disrupt fluid homeostasis in the small intestine. This results in mild to severe diarrhea within a few days post-weaning, associated with clinical signs of dehydration, loss of body condition (i.e. disappearance of muscle volume) and mortality [9]. The adhesive fimbriae most commonly occurring in ETEC from pigs with PWD are F4 (K88) and F18 [9]. Other fimbriae such as F5 (K99), F6 (987P) and F41 rarely occur in *E. coli* isolates from PWD [9-13]. The main enterotoxins associated with porcine ETEC are heat-labile toxin (LT), heat-stable toxin a (STa) and heat-stable toxin b (STb). In some cases, both enterotoxins and a Shiga toxin (Stx2e) are produced by the pathogenic stains [9].

The disease is currently controlled using antimicrobials, although the emergence of antimicrobial resistance in *E. coli* strains isolated from cases of PWD urges the need for alternative control measures [14-18]. Several alternative

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strategies have been explored to increase intestinal health and to decrease incidence of PWD due to *E. coli* in post-weaned piglets [19-21]. Overall, inclusion of additional dietary fiber and reduction of crude protein levels in post-weaning diets seem to be an effective nutritional strategy that may counteract the negative effects of protein fermentation in the pig gut [20, 22-24]. Although it has been reported that specific fermentable carbohydrates combined with reduced crude protein content have altered the microflora and fermentation patterns in the gastro-intestinal tract of post-weaned piglets; these favorable effects do not necessarily result in increased growth performance [25]. Other feeding strategies have been more focused on feed consistency, thereby feeding more coarsely ground meal to the post-weaned piglets [26]. Coarsely ground feed meals change the physico-chemical conditions in the stomach, thereby increase the concentrations of organic acids which can lower the pH. This promotes growth of anaerobic lactic acid bacteria and reduces the survival of *E. coli* during passage through the stomach [26]. Fermentation of undigested dietary protein and endogenous proteins in the large intestines yield putative toxic metabolites that can impair epithelial integrity and promote enteric disorders such as PWD [27]. Incidence and severity of PWD may also be influenced by addition of probiotics to the diet, which may change the fermentation profile and thus promote gut health [28]. Furthermore, medium chain fatty acids (MCFAs) can neutralize bacterial metabolites in the small intestine [29].

Since the late 1980's, several studies on zinc supply to post-weaned piglets have been performed. Several nutritional studies have demonstrated the effects of dietary zinc oxide (ZnO) in the prevention and healing of PWD [30]. Therefore, ZnO has been admitted in the prevention and control of PWD at levels, up to 3,000 parts per million (ppm) through the feed for a maximum of 14 days post-weaning. However, the Committee for Veterinary Medicinal Products (CVMP) has recently decided that the use of ZnO in post-weaning diets should be phased out, at the latest by 2022 throughout the EU [31].

Therefore, other preventive strategies have recently been explored [1,32]. For an *E. coli* vaccination against PWD due to F4-ETEC and F18-ETEC, the prerequisite is that active mucosal immunity against F4 and F18 is mounted. This implies the local production of F4-ETEC and/or F18-specific secretory IgA antibodies, which prevent pathogenic F4-ETEC and F18-ETEC to attach to the intestinal F4- and F18-receptors and thus reduce the clinical signs of PWD [32]. Recently, vaccination with a live non-pathogenic *E. coli* F4 or *E. coli* F4 and F18 vaccine has demonstrated efficacy against PWD due to F4-ETEC, and F4-ETEC and F18-ETEC [33,34]. Immunizations against the F4-ETEC and F18-ETEC pathogens have resulted in decreased severity and duration of PWD clinical signs and fecal shedding of F4-ETEC and F18-ETEC [33,34]. Moreover, increased weight gain has been demonstrated in piglets vaccinated with *E. coli* F4 vaccine [33].

Here, we report results demonstrating the efficacy of an oral live non-pathogenic *E. coli* F4 vaccine (Coliprotec® F4; Elanco; Greenfield, IN, USA) for active immunization of piglets against PWD caused by F4-ETEC with different feeding strategies, combining normal and/or high energy and protein diets in a 3-phase approach with or without additional nutraceuticals under field conditions. We also included a group using the current approach of 3,000 ppm ZnO during 14 days post-weaning and a group with addition of a nutraceutical concept containing MCFAs, organic acids and additional fibers.

MATERIALS AND METHODS

Experimental Farm Description

The field trial was performed on a conventional farrow-to-finish pig farm with 600 DanBred sows in Flanders (Belgium). The farm was managed in a 4-week batch-management system (with alternately weaning) with 120 sows per production batch. This management approach has been shown to improve the health status for several respiratory pathogens [35]. Piglets were weaned at 23 days of age and housed in specifically equipped post-weaning facilities, where they were raised for 7 weeks (50 days post-weaning). The post-weaning facility was equipped with 40 pens, which could each house 16 post-weaned piglets. Dry feeders with two waterers, one on each side, were located at the pen division, thus feeding two pens with a total of 32 piglets. The pens were further equipped with fully slatted plastic floors and were heated with hot water tubes on the side walls near the air inlet. Ventilation was performed through 3 ventilation tubes and fresh air entered into the compartment directly from the outside.

ETEC Diagnosis and Characterization at Experimental Farm

The farm was selected following ETEC diagnostics during the post-weaning period. Therefore, untreated piglets (n = 10) with typical clinical signs of PWD, such as watery feces, thin belly and signs of dehydration, were sampled using rectal swabs (Sterile Transport Swab Amies with Charcoal medium; Copan Italia S.p.A., Brescia, Italy). All sampled piglets were between 3 and 5 days post-weaning. The diagnostic samples were sent to the laboratory (IZSLER, Brescia, Italy) under cooled conditions for further processing.

Specimen were processed using standard procedures for isolation and characterization of intestinal *E. coli* [18]. Briefly, samples were plated on selective media and on tryptose soy agar medium supplemented with 5% of defibrinated ovine blood and incubated aerobically overnight at 37°C. Haemolytic activity was evaluated and single coliform colonies were further characterized.

DNA samples were prepared from one up to five haemolytic and/or non-haemolytic *E. coli* colonies and used to perform a multiplex PCR for the detection of fimbrial and toxin genes, including those encoding for F4 (K88), F5 (K99), F6 (987P), F18, F41, LT, STa, STb and Stx2e, but not discriminating between F4ab, F4ac and F4ad. The methodology used for the identification of these virulence genes has been described previously [36].

All collected samples were positive for F4 in combination with STa, STb and LT. No other virulence factors could be detected.

Vaccination with a Live Non-Pathogenic *E. coli* F4 Vaccine

In order to vaccinate piglets at least 7 days before the clinical signs to mount sufficient protective local immunity in the gut [33], piglets were vaccinated at 18 days of age (5 days prior to weaning), during the suckling period. The live non-pathogenic *E. coli* F4 vaccine has a rapid onset of immunity (7 days) and a duration of immunity of 21 days post-vaccination [33], which covers the most critical period of PWD [1]. An efficacy trial using an experimental *E. coli* F4 challenge at 3 days post-vaccination showed reduction of the severity and duration of PWD and reduction in fecal shedding of pathogenic F4-ETEC [33]. Sows were randomly assigned to treatment (pathogenic *E. coli* F4 vaccine) or control group based on their

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parity and sow number. Parities were equally distributed to both treatment groups. Piglets from sows assigned to the treatment group were vaccinated orally through drenching with 2 ml of a live non-pathogenic *E. coli* F4 vaccine. Piglets from sows in the control group were not treated nor vaccinated. No antibiotics were administered to piglets from 15 days of age onwards, in order to omit interference with the development of protective local immunity by the *E. coli* F4 vaccine during the 7 days following vaccination.

Table 1. Schematic description of experimental trial set-up including treatment groups and their short comprehensive description and the respective differences in feeding strategies (weaning starter, starter and grow starter; blocks with the same F* code have identical compositions), addition of ZnO (3,000 ppm), supplementary nutraceuticals (MCFAs, organic acids and additional fibers) and vaccination with a live non-pathogenic *E. coli* F4 vaccine.

	Treatment groups				
	A	**B**	**C**	**D**	**E**
Treatment description	Normal + ZnO	Normal + nutraceuticals	Normal start / High 2nd & 3rd phase	High 3-phases	High 3-phases + nutraceuticals
Weaning starter	3 kg F1*	3 kg F1*	3 kg F1*	3 kg F2**	3 kg F2**
Starter	5 kg F3*	5 kg F3*	5 kg F4**	5 kg F4**	5 kg F4**
Grow starter	… kg F5*	… kg F5*	… kg F6**	… kg F6**	… kg F6**
ZnO (14d)	3,000 ppm	0	0	0	0
Nutraceuticals	0	2 kg / tonne	0	0	2 kg / tonne
E. coli F4 vaccine	no	no	yes	yes	yes

* F1, F3 and F5 are diets with the normal energy and protein levels.
** F2, F4 and F6 are diets with an increased energy and protein levels.

Experimental Design

At weaning, *E. coli* vaccinated piglets were randomly assigned to three groups with a different feeding strategy. The unvaccinated control piglets were randomly assigned to two groups with different preventive measures supplemented to the feed against PWD due to *E. coli*. Each treatment group consisted of 128 piglets divided over 8 pens with 16 piglets each. Sexes were distributed equally within and between different treatment groups. The treatment groups were randomly allocated to the different pens within the compartment in order to evenly distribute all treatments for potential interaction with specific climatic subzones within the compartment (outer walls, air inlet, central part). Details on the experimental design in relation to feeding strategies and preventive measures are given in Table 1. Piglets were weighed per pen (n = 16 piglets) at three different time-points: d0 (start), d21 (mid-term) and d50 (end). Average piglets weights were calculated based on pen weight and number of piglets present at the moment of weighing. Piglet treatment identification was blinded to both farmer and veterinarian involved in trial follow-up by letter codes (A, B, C, D, and E).

Feeding Strategies

Feeding strategies were based on previous results [37], showing that a 3-phase feeding strategy has proven the most optimal production results. Additionally, different combinations of normal or high energy and protein diets in the 3-phase approach were tested together with *E. coli* F4 vaccination and addition of 2 kg per tonne of extra protective nutritional supplements, i.e. nutraceuticals, consisting of a combination of MCFAs, organic acids and additional fibers. All analytical feed compositions are given in detail in Table 2. Unvaccinated control groups were also fed the 3-phase feeding strategy. One unvaccinated group was designed to resemble the current field situation with addition of 3,000 ppm ZnO to the feed during the first 14 days post-weaning, whereas the other unvaccinated group was formulated with 2 kg per tonne of nutraceutical supplement.

Treatment

No group treatments were performed during the entire study period. Individual piglets with severe clinical signs of PWD were treated with an injectable antimicrobial, i.e. lincomycin. Other disorders were treated by the farmer, following consultation of the veterinarian, with the appropriate antimicrobial where needed. All individual treatments were registered with date, pen, product type and reason for treatment.

Performance parameters

The following performance parameters were collected during the trial: piglet weight at d0, d21 and d50, feed intake during period 1 (0-21 days), period 2 (21-50 days) and period 3

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(0-50 days), individual treatments with specific reason for treatment, mortality with date of death (number of days in trial) and piglet weight. Average daily weight gain (ADWG) was calculated based on piglet weight and number of days in trial for period 1 (0-21 days), period 2 (21-50 days) and period 3 (0-50 days). Feed conversion rate (FCR), the amount of feed to add one kg of bodyweight, was calculated based on average daily weight gain and feed intake for period 1 (0-21 days), period 2 (21-50 days) and period 3 (0-50 days). Treatment incidence 50 (TI_{50}) was calculated based on the number of individual injections per treatment for a total of 100 piglets over the trial duration of 50 days.

Table 2. Analytical feed composition of the feed formulations in the 3-phase feeding strategy, with in each phase a normal dietary composition and a high energy and protein composition.

	Phase 1		Phase 2		Phase 3	
Feed code	F1	F2	F3	F4	F5	F6
Energy/protein level	normal	high	normal	high	normal	high
NE pig (kcal/kg)	2421	2530	2389	2447	2349	2405
Crude protein (g/kg)	167	190	172	189	170	186
Crude fat (g/kg)	60	76	60	68	54	61
Sugar/starch (g/kg)	419	397	406	394	406	396
Crude ashes (g/kg)	48	49	47	48	46	46
Crude fiber (g/kg)	44	40	44	43	45	42
Digestible lysine (g/kg)	10.5	12.0	10.5	11.6	10.2	11.3

Pen Fecal Clinical Score and General Clinical Score

Piglet feces consistency was scored daily from d0 to d21 using pen fecal clinical score (FCS) as described in Table 3. FCS was performed by the same person throughout the entire duration of the trial observation (0-21 days). Piglets were also scored on general appearance using a general clinical score (GCS), ranging from 0 (= severe clinical condition) to 10 (= excellent clinical condition). For both pen FCS and GCS, one score per pen was attributed daily in the morning at 9 am. For analysis, area under the curve (AUC) and time to maximal score was calculated per pen for both pen FCS and GCS. Clinical assessment of piglets with diarrhea was performed based on appearance of fluid watery stools in the anal and perineal region. The number of piglets per pen with these clinical signs was counted daily from d0 to d21 and reported as total number of piglets with diarrhea per treatment group over the entire observation period (0-21 days).

Table 3. Comprehensive description of the pen fecal clinical score with its interpretation and the clinical aspect of the fecal clinical score.

Score	Interpretation	Clinical aspect
0	Normal	Normal fecal consistency
1	Pasty	Soft pasty consistency
2	Mild	Presence of fluid, but more particles than fluid
3	Moderate	More fluid than particles
4	Severe	Fluid watery feces

Statistical Analysis

For the continuous data, effect of treatment was assessed using pairwise comparison using t-test with pooled standard deviations. For the ordinal outcomes, effect of treatment was assessed using pairwise comparison using Wilcoxon rank sum test. The P-values were adjusted with the Bonferroni method for multiple comparison. All tests were performed at the nominal level of 5%.

RESULTS

Piglet Weight and Average Daily Weight Gain

On d0, average individual piglet weight was not significantly different among treatment groups, indicating an equal starting weight in all groups. At the mid-point weighing (d21), group A (ZnO) had a non-significantly higher ($P > 0.05$) weight as compared to the other treatment groups. At d50, group B (nutraceuticals) had the lowest numerical average individual piglet weight at d50, followed by group A (ZnO), although no significant differences ($P > 0.05$) were present between all treatment groups (Fig.1).

For period 1 (0-21 days), group B (nutraceuticals) had a non-significantly lower ($P > 0.05$) ADWG as compared to the other treatment groups. As expected from previous trials, group A (ZnO) had the highest, though not significant, ADWG as compared to the other treatment groups. The piglets vaccinated with the *E. coli* F4 vaccine grew equally well, although the groups D and E with the high energy and protein diets from the 1st phase onwards had a slightly higher ADWG as compared to group C that was fed a normal energy and protein diet in the 1st phase. For period 2 (21-50 days), no significant differences ($P > 0.05$) among treatment groups were observed. Nevertheless, *E.*

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coli F4 vaccinated groups (C, D and E) has a numerically higher ADGW in the 2nd period as compared to group A (ZnO) and group B (nutraceuticals). Overall ADWG (0-50 days) significantly differed ($P \leq 0.05$) between group B (nutraceuticals) and the different treatment groups (Fig. 2).

Feed Conversion Rate

For period 1 (0-21 days) and period 2 (21-50 days), no significant differences among different treatment groups could be observed. Nevertheless, FCR in period 1 (0-21 days) was numerically higher ($P > 0.05$) in group B (nutraceuticals) as compared to group A (ZnO) and group E (high energy and protein + nutraceuticals). In period 2 (21-50 days), all *E. coli* vaccinated groups (C, D and E) had a numerically lower ($P > 0.05$) FCR as compared to both unvaccinated groups (A and B). Overall FCR (0-50 days) was significantly higher ($P \leq 0.05$) in group B (nutraceuticals) as compared to group D (high energy and protein), whereas FCR in all other groups was not significantly different ($P > 0.05$) from each other.

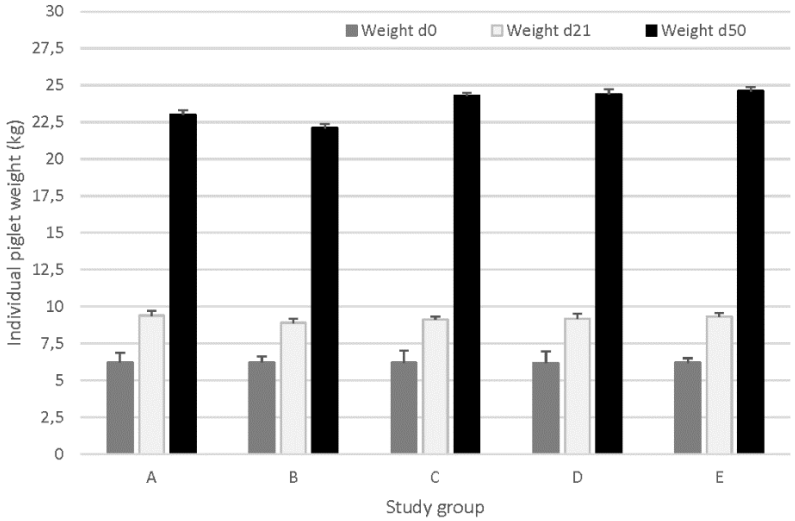

Fig. 1. Average individual piglet weight (expressed in kg; mean ± SEM) for piglets at d0 (start of trial), d21 (mid-point weighing) and d50 (end of trial). Different treatment groups differed in diets composition (normal or high energy and protein levels) and preventive approach towards post-weaning diarrhea (ZnO, nutraceutical or vaccination against *E. coli* F4). Group A was fed a diet with normal energy and protein levels and supplemented with 3,000 ppm ZnO for the first 14 days post-weaning. Group B was was fed a diet with normal energy and protein levels and supplemented with additional nutraceuticals (2 kg / tonne). Group C, D, and E were vaccinated with Coliprotec® F4 at 18 days of age. Group C was fed a starter diet (1st phase) with normal energy and protein levels followed by a 2nd and 3rd phase with higher energy and protein levels. Group D and E were fed 3-phases with high energy and protein levels. Group E was additionally supplemented with nutraceuticals (2 kg / tonne). Different superscript letters indicate statistically significant differences ($P \leq 0.05$).

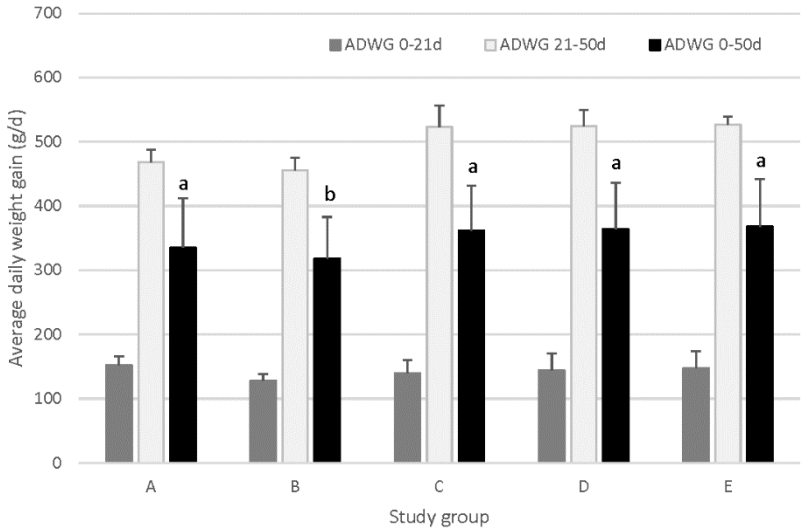

Fig. 2. Average daily weight gain (ADWG; expressed in g/d; mean ± SEM) for piglets during period 1 (0-21 days post-weaning), period 2 (21-50 days post-weaning) and period 3 (0-50 days post-weaning). Different treatment groups differed in diets composition (normal or high energy and protein levels) and preventive approach towards post-weaning diarrhea (ZnO, nutraceutical or vaccination against *E. coli* F4). Group A was fed a diet with normal energy and protein levels and supplemented with 3,000 ppm ZnO for the first 14 days post-weaning. Group B was was fed a diet with normal energy and protein levels and supplemented with additional nutraceuticals (2 kg / tonne). Group C, D, and E were vaccinated with Coliprotec® F4 at 18 days of age. Group C was fed a starter diet (1st phase) with normal energy and protein levels followed by a 2nd and 3rd phase with higher energy and protein levels. Group D and E were fed 3-phases with high energy and protein levels. Group E was additionally supplemented with nutraceuticals (2 kg / tonne). Different superscript letters indicate statistically significant differences ($P \leq 0.05$).

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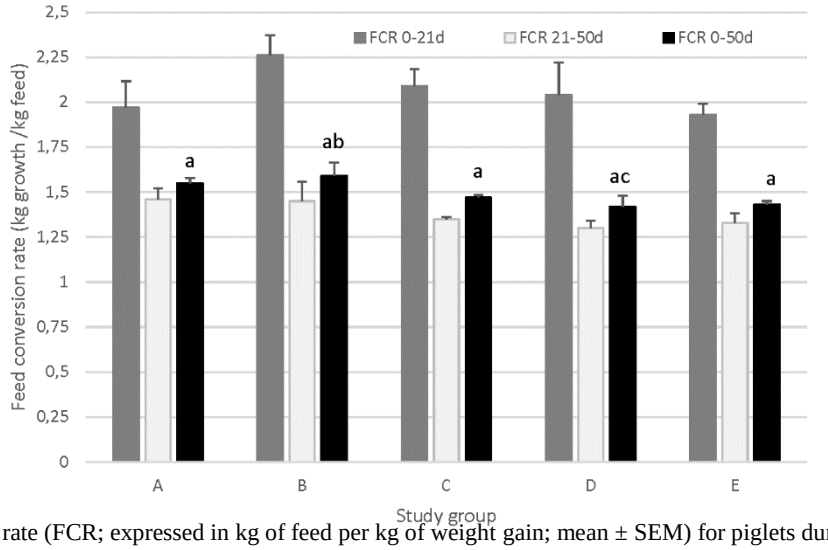

Fig. 3. Feed conversion rate (FCR; expressed in kg of feed per kg of weight gain; mean ± SEM) for piglets during period 1 (0-21 days post-weaning), period 2 (21-50 days post-weaning) and period 3 (0-50 days post-weaning). Different treatment groups differed in diets composition (normal or high energy and protein levels) and preventive approach towards post-weaning diarrhea (ZnO, nutraceutical or vaccination against *E. coli* F4). Group A was fed a diet with normal energy and protein levels and supplemented with 3,000 ppm ZnO for the first 14 days post-weaning. Group B was was fed a diet with normal energy and protein levels and supplemented with additional nutraceuticals (2 kg / tonne). Group C, D, and E were vaccinated with Coliprotec® F4 at 18 days of age. Group C was fed a starter diet (1st phase) with normal energy and protein levels followed by a 2nd and 3rd phase with higher energy and protein levels. Group D and E were fed 3-phases with high energy and protein levels. Group E was additionally supplemented with nutraceuticals (2 kg / tonne). Different superscript letters indicate statistically significant differences ($P \leq 0.05$).

Pen Fecal Clinical Score and General Clinical Score

Pen FCS was collected daily for each individual pen from 0 to 21 days post-weaning. Pen FCS, expressed as AUC, was significantly higher ($P \leq 0.05$) in group B (nutraceuticals) as compared to group C (low/high/high energy and protein), whereas pen FCS in all other groups was not significantly different ($P > 0.05$) from each other. Overall, pen FCS was comparable between group A (ZnO), which is considered the standard approach, and the *E. coli* vaccinated groups (Table 4). Although some numerical differences in time to maximal FCS occurred among different treatment groups, no significant differences ($P > 0.05$) could be observed in the time to maximal FCS (Table 4).

The number of piglets with clinical signs of diarrhea was significantly higher ($n = 151$; $P \leq 0.05$) in group B (nutraceuticals) and group D (high energy and protein) as compared to group A (ZnO) and two of the *E. coli* vaccinated groups, namely group C (low/high/high energy and protein) and group E (high energy and protein + nutraceuticals). The number of piglets with clinical signs of diarrhea was also significantly different ($P \leq 0.05$) between group B (nutraceuticals) and group D (high energy and protein) (Table 4).

Pen GCS was collected daily for each individual pen from 0 to 21 days post-weaning. AUC of pen GCS was not significantly different ($P > 0.05$) among the different treatment groups (Table 4). Although some numerical differences in time to maximal GCS occurred between the different treatment groups, no significant differences ($P > 0.05$) could be observed (Table 4).

Mortality

Data related to mortality are given in Table 5. In summary, mortality remained low in all treatment groups varying between 0 and 1.56%. A total of two piglets died in Period 1 (0-21 days) with one occurring in group A (ZnO) on d18 and one occurring in group B (nutraceuticals) on d12. All other mortality cases were registered in the period 2 (21-50). In group D (high energy and protein) no piglets died during the entire trial period. Average weight of dead piglets was 5.75 kg in period 1 (0-21 days) and 5.10 kg in period 2 (21-50 days). This clearly indicates that mortality in period 2 (21-50 days) was mainly due to runt piglets, except for group A (ZnO) where the dead piglet weighed more than 9 kg.

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Table 5. Mortality results per treatment group and study period with number of dead piglets per group (percentage of total piglets enrolled in the group), weight of the dead piglets (kg), and day of post-weaning mortality (d).

Treatment group	Study period					
	Period 1 (0-21 d post-weaning)			Period 2 (22-50 d post-weaning)		
	Mortality - number (%)	Average weight dead piglets (kg)	Average days post-weaning (d)	Mortality - number (%)	Average weight dead piglets (kg)	Average days post-weaning (d)
A	1 (0.78%)	7.00	18.0	1 (0.78%)	9.50	29.00
B	1 (0.78%)	4.50	12.0	1 (0.78%)	5.00	44.00
C	0 (0.00%)	N/A	N/A	2 (1.56%)	3.50	43.00
D	0 (0.00%)	N/A	N/A	0 (0.00%)	N/A	N/A
E	0 (0.00%)	N/A	N/A	1 (0.78%)	4.00	42.00

DISCUSSION

Based on the current study, we can conclude that active immunization of piglets against PWD caused by F4-ETEC performed comparable to the standard approach under field conditions with addition of 3,000 ppm ZnO during the first 14 days post-weaning. Average individual piglet weight at 21 days post-weaning was equal in all treatment groups. Nevertheless, piglets vaccinated with the *E. coli* F4 vaccine were numerically heavier (1.3 to 1.6 kg extra) at the end of the nursery period (d50) as compared to piglets in group A (ZnO). This is in accordance with a previous study demonstrating the same effect of ZnO supplementation during 14 days post-weaning on piglet performances [37]. Under field conditions, an extra kg of piglet weight during the nursery period is considered to result in at least 2-3 kg extra weight during the fattening period. This implies earlier slaughter at the same weight or heavier fattening pigs at the same slaughter age. Both scenarios imply economic benefit to the swine farmer. Average daily weight gain behaved in the same trend, although the ADWG for period 2 (21-50 d) was numerically lower in the ZnO-supplemented group (A). This is in accordance with a previous study where ADWG in the ZnO-supplemented group was significantly lower as compared to the *E. coli* vaccinated groups in period 2 (21-50 days) [37]. Under field conditions, most farmers only have access to start- and end-point data related to post-weaning performances, therefore the numerically higher mid-term performance in the ZnO-supplemented group (A) is not considered relevant to practice. Nevertheless, the higher weight indicate that piglets supplemented with ZnO at 3,000 ppm for 14 days post-weaning might have a stable intestinal integrity and pathogenic *E. coli* bacteria have less impact on the performance of these piglets during the early post-weaning phase [30]. However, CVMP has recently decided that the use of ZnO in post-weaning diets should be phased out, at the latest by 2022 throughout the EU [31]. Therefore, alternative approaches to control PWD due to pathogenic *E. coli* should be explored. In the current study, *E. coli* F4 vaccination combined with diets containing a higher level of energy and protein resulted in piglet performances (weight, ADWG and FCR) that reached levels equal to the current standard of ZnO supplementation under field conditions. Therefore, *E. coli* F4 vaccination might be one of the alternative strategies once ZnO is banned in the EU by 2022. Besides vaccination, several other alternative strategies, such as adapted nutritional strategies (feed consistency, lower crude protein, digestible fibers and other dietary fibers), prebiotics, probiotics, organic acids, MCFAs, specific IgA antibodies and oral vaccination have been explored [19-29,33,34,38-41].

In the current study, a nutraceutical approach, including a mixture of MCFAs, organic acids and additional fiber, was evaluated. Although performance parameters (weight, ADWG and FCR) were in line with the *E. coli* vaccinated groups and supplementation of ZnO, other parameters related to health (pen FCS and TI_{50}) were significantly worse, indicating this approach did not provide as much protection as ZnO supplementation or *E. coli* F4 vaccination. Indeed, intestinal pathogens have many different mechanisms to interact with the host, which makes complete inhibition of their pathogenesis through specific feed additives or combination of these additives quite challenging [21,42]. However, when combining the nutraceutical supplementation with an *E. coli* F4 vaccination in a high energy and protein 3-phase feeding strategy, piglet performances were numerically better as compared to the group without the nutraceuticals. Morever, pen FCS were also better and the number of piglets with clinical signs of PWD decreased when nutraceuticals were added to the high energy and protein diets. This indicates that under conditions of additional protection with an *E. coli* vaccine, the nutraceutical could support general gut health in order to improve clinical condition of the supplemented piglets.

Recently, vaccination with a live non-pathogenic *E. coli* F4 or *E. coli* F4 and F18 vaccine has demonstrated efficacy against PWD due to F4-ETEC, and F4-ETEC and F18-ETEC [33,34]. Immunization against the F4-ETEC and F18-ETEC pathogens resulted in decreased severity and duration of PWD clinical signs and fecal shedding of F4-ETEC and F18-ETEC [33,34]. Moreover, increased weight gain was demonstrated in piglets vaccinated with *E. coli* F4 vaccine [33]. Our results are in line with these observations, indicating that feeding strategy with an

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increased content of energy and protein had no negative impact on results induced by immunization with an *E. coli* F4 vaccine under field conditions. This implies that farms suffering from PWD due to F4-ETEC can still explore alternative or more challenging feeding strategies that results in higher piglet performances when piglets are concurrently protected through immunization with an *E. coli* F4 vaccine. This is in contrast with current belief that higher energy and protein diets induce a higher risk for PWD. Indeed, without additional vaccination, this might be a reality, resulting in a higher pen FCS and more piglets showing clinical signs of diarrhea. However, with the current strategy of vaccination, the risk for more pronounced signs of PWD could be mitigated. Although pen FCS and number of piglets with clinical diarrhea were higher in group D (high energy and protein) and group E (high energy and protein + nutraceuticals) as compared to group C (low/high/high energy and protein), which was fed a normal diet in the 1st phase, this did not result in decreased piglet performances or increased medication (TI_{50}) and mortality.

As expected, supplementation of ZnO resulted in the acceptable pen FCS and TI_{50} although time to maximal fecal clinical score did not differ among treatment groups. Nevertheless, from 14 days post-weaning onwards, at removal of the ZnO from the feed, pen FCS increased again, in contrast to the other groups, where pen FCS remained stable during that specific period. In practice, this phenomenon is referred to as 'post-ZnO diarrhea' and sometimes even needs antibiotic treatment to control. *E. coli* vaccinated piglets had similar pen FCS and GCS, which remain important evaluation parameters in practice, due to lack of many other directly available data for evaluation of preventive or clinical interventions to prevent or control PWD due to *E. coli*.

Another important evaluation parameter to assess the success of different intervention strategies in relation to PWD due to *E. coli* is mortality [33]. Mortality data were low and did not significantly differ among treatment groups. In the current study, mortality remained within the acceptable level of 2% during the post-weaning period, indicating that the different intervention strategies could protect the piglets against PWD due to pathogenic *E. coli* under field conditions. This is in contrast to a previous study [37], where mortality in the control group was elevated to as high as 12.5% and additional antibiotic intervention through individual injection had to be performed.

In conclusion, the present study demonstrated the efficacy of Coliprotec® F4 oral live non-pathogenic vaccine for active immunization of piglets against PWD due to F4-ETEC. Different feeding strategies had no significant impact on the clinical outcome, although performance parameters of these vaccinated piglets numerically differed. Increased levels of energy and protein from the 1st phase onwards resulted in better overall performances, although piglets with a normal diet in phase 1 did not perform significantly inferior over the entire post-weaning period. In many parameters, *E. coli* vaccination performed comparable to the ZnO-supplemented group. However, this approach is no longer future-proof due to EU-regulations on total ban of ZnO by 2022. Therefore, *E. coli* vaccination could be one of the preventive options to protect piglets against PWD due to *E. coli* in the near future. In contrast, the alternative strategy combining MCFAs, organic acids and additional fibers resulted in significant clinical diarrhea, requiring additional antibiotic treatment to control the disease. Nevertheless, in the nutraceutical-supplemented group, other performance parameters were similar to *E. coli* vaccination or ZnO supplementation.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of intrests.

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