Multisite Controlled Blinded Field Studies to Evaluate the Effect of an Oral Live Escherichia Coli Bivalent F4/F18 Vaccine in Pig Nurseries with Mild Cases of Post-Weaning Diarrhea or Subclinical ETEC Infections.

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Abstract

Background: Post-weaning diarrhea (PWD) caused by *Escherichia coli* is a worldwide economically important disease in pigs, linked with increased mortality and weight loss. F4- and F18-enterotoxigenic *E. coli* (ETEC) are the most diagnosed causes of PWD. The efficacy of live oral *E. coli* vaccines has been previously demonstrated on farms with confirmed clinical cases of PWD. However, the impact of vaccination in subclinical cases with mild or not noticeable clinical manifestations needed to be assessed. This study evaluated the effect of an oral dose of a live *E. coli* bivalent F4/F18 vaccine in nurseries without ongoing clinical PWD outbreaks but with a history of pathogenic *E. coli* circulating within the herd. Two controlled blinded studies were performed under commercial conditions in a total of 6 European sites. Overall, 1941 vaccinated piglets and 1976 control piglets were enrolled and were monitored for health, growth, and feed conversion rate.

Results: No clinical outbreak of ETEC PWD has been observed in the tested farms. Two farms faced episodes of non-ETEC enteric diseases that required treatments. Nursery weight gain was not statistically different between groups in these two farms. However, the nursery weight gain of the vaccinated pigs was significantly higher by 0.80, 0.87 and 1.24 kg than of the control pigs in three of the four farms without non-ETEC enteric diseases ($p < 0.05$) and by 0.69 kg ($p = 0.051$) in the fourth one, leading to numerically heavier pigs at the exit of the four nurseries. No statistically difference was observed for the feed conversion rate, though it was numerically lower for vaccinated pigs of the two tested farms.

Conclusions: Recent European legislations aimed at reducing the use of antibiotics and banning therapeutic use of zinc oxide in feed are leading to a search for alternatives to compensate animal health impacts and economic losses due to PWD. These studies demonstrate that, even in the absence of convincing clinical signs of PWD, ETEC infections limit nursery pig optimal growth performance that can be mitigated by vaccination with an oral live *E. coli* bivalent F4/F18 vaccine. These findings corroborate previous studies showing the effect of vaccination in clinical outbreaks of PWD.

Background

Escherichia coli (*E. coli*) related post-weaning diarrhea (PWD) is a worldwide economically important disease in pigs due to increased mortality, weight loss and heterogeneity, slow growth, and additional treatment costs (Luppi et al., 2016; Lyutskanov, 2011; Fairbrother and Nadeau, 2010; Svensmark et al., 1989). PWD is mainly caused by enterotoxigenic *E. coli* (ETEC) that express F4 (K88) or F18 adhesive fimbriae mediating the colonization of the intestinal mucosa and producing enterotoxins that induce secretory diarrhea (Fairbrother and Nadeau, 2019). PWD is typically described as a disease occurring in the first weeks after weaning characterized by sudden death or by diarrhea, dehydration, marked reduction in feed consumption and growth retardation in surviving piglets (Fairbrother and Nadeau, 2019; Rhouma et al., 2017). Additionally, ETEC infections with evident colonization of the pig intestine but without noticeable diarrhea and mortality were sparsely reported and were associated with lower growth performance after weaning (Pollock et al., 2019; Athanasiadou et al., 2010; Hampson, 1994).

For decades, antibiotics, particularly colistin, in combination with therapeutic levels of zinc oxide in the feed were widely used to control PWD across Europe and globally. The emergence of antimicrobial resistance among *E. coli* isolates (Rhouma et al., 2017; Jahanbakhsh et al., 2016; Luppi et al., 2015) and the recent European adopted measures to significantly reduce the use of colistin in animals (EMA, 2016) and to ban the therapeutic use of zinc oxide in feed (EMA, 2017) highlight the need for alternative control strategies.

An oral live *E. coli* bivalent F4/F18 vaccine against PWD caused by F4- and/or F18-ETEC (Coliprotec® F4/F18, Prevtex Microbia GmbH, Germany) is available in Canada and the European Union. It has been demonstrated that the use of this oral live *E. coli* vaccine, both in farms with confirmed clinical cases of PWD and in controlled ETEC challenge studies, improved the health of pigs and reduced ETEC intestinal colonization, ETEC fecal shedding, need for antibiotic treatments and weight loss due to F4- and/or F18-ETEC infection (Meymerit et al., 2019; Gibellini and Perro, 2019; Fily et al., 2019; Vangroenwegue and Bonny, 2018; Vangroenwegue and De Braekeleer, 2018; Vangroenwegue et al., 2018; Piqué et al., 2018; Vangroenwegue, 2017; Sánchez Uribe et al., 2017; Nadeau et al., 2017; Gibellini et al., 2017; Fairbrother et al., 2017; Nadeau et al., 2016). Hence, oral vaccination of pigs with a live *E. coli* vaccine against F4- and F18-ETEC represents a sustainable, practical, and effective approach in such clinical situations.

The impact of vaccination of herds with subclinical PWD cases, where the load of ETEC in the pig intestine is low, resulting in mild clinical outcomes or not clinically noticeable cases, needed to be assessed. This study aimed to evaluate the effect of an oral live *E. coli* bivalent F4/F18 vaccine in pig nurseries without major outbreaks of PWD but with a history of pathogenic *E. coli* circulating within the herd. The hypothesis was that although the pigs appear healthy, the subclinical ETEC infection impairs their growth performance and vaccination with an oral live *E. coli* bivalent F4/F18 vaccine would reduce the negative impact like weight loss, slow growth and weight heterogeneity due to the ETEC presence in the intestine.

Methods

Animals and trial design

Two controlled, randomized, blocked and blinded clinical field studies were performed under commercial conditions at multiple sites in Europe according to Good Clinical Practice (GCP VICH GL9). The studies compared a group of piglets vaccinated with one oral dose of the live *E. coli* bivalent F4/F18 vaccine (Coliprotec® F4/F18, Prevtex Microbia GmbH, Germany) with a non-vaccinated control group of piglets. The vaccine was prepared with a solution of 5 g of skimmed milk powder per liter of water; the control piglets received the skimmed milk solution only. The skimmed milk was used to mask the treatment and to neutralize chlorine or other sanitizers that could be present in the water. Pigs were monitored until the end of the nursery period (flatdeck). Nurseries refer to facilities that house newly weaned pigs until they reach the grower/finisher stage.

The first study, performed in 2017, included one production batch for each of the 3 German farms tested (sites GA, GB and GC). The second study took place between 2016 and 2018 and comprised 2 German farms (sites GD and GE) and one Danish farm (site DA). Two consecutive production batches were enrolled for GD and DA. The trial was stopped after one production batch in GE due to an outbreak of porcine parvovirus. Details of study sites are presented in the Table 1. All selected farms were considered at risk of mild *E. coli* episodes based on historical data of the previous 6 months. This risk was more obvious on GC for which cases of PWD characterized by low prevalence of pigs showing mild diarrhea due to F4-ETEC were diagnosed in weaned batches prior the study.

The studies were blinded at each site by using separate teams to administer the vaccine/control product and to perform clinical examinations. Procedures were in place to avoid cross-vaccination between groups: separate access to the production units, specific clothes, gloves and shoes that were changed between groups, and dedicated equipment whenever possible, otherwise the equipment was thoroughly cleaned and disinfected between groups.

The number of pigs enrolled per study site and production batch are presented in Table 1. Inclusion criteria comprised piglets derived from sows not vaccinated against *E. coli*, physically healthy and at least 18 days of age at the day of vaccination (day 0). Animals moribund at day 0 or that received antimicrobials active against *E. coli* from day - 3 to day 0 were not included into the study. A total of 2242 piglets (1109 vaccinated and 1133 control) and 1675 piglets (832 vaccinated and 843 control) were enrolled at day - 1 ± 1 for the first and second study, respectively.

The experimental timeline and approximate age of the pigs are presented in Fig. 1. Piglets of GA were vaccinated using bowls at the day of weaning (aged 20 ± 1 days). Dosing on GB and GC was done in the farrowing unit by oral drenching at a similar age as GA's piglets and were weaned at approximately 28 and 25 days, respectively. Pigs of GD, GE and DA were vaccinated in the farrowing unit using bowls at 20 ± 2 days of age and then weaned at approximately 24, 26 and 30 days of age, respectively (Table 1). Treatment groups of GA were balanced by sex and body weight before treatment on day 0. For piglets of GB, GC, GD, GE and DA, mother sows, i.e. the complete litter, were allocated to study groups based on a pre-established randomization list as vaccination was done prior to weaning.

The studies terminated at each farm at the end of the nursery period at the study day 48, 53 and 42 for GA, GB and GC, and at the study day 48, 78 and 50 for the sites GD, GE and DA (Table 1 and Fig. 1).

Table 1
Details of the study sites and number of pigs enrolled

Study	Study site	Location	Type of farm ¹	No. of sows	No. of nursery places	No. of pigs per unit	Pig genetic line	Weaning age (~ days of life)	Study length (days)	Vaccinated pigs (n)	Control pigs (n)
First	GA	North Germany	FN	550	2700	32	BHZP	21	48	383	382
	GB	South Germany	FF	450	2100	100	Danish Landrace	26–28	53	361	362
	GC	South Germany	FN	500	3000	40	Danish Landrace	25	42	365	389
Second	GD	North Germany	FF	260	1200	200	Topigs and Pietrain	24	48	Batch 1: 209 Batch 2: 226	Batch 1: 213 Batch 2: 229
	GE	South Germany	FF	100	560	42	DL and Pietrain x Duroc	26	78	97	93
	DA	Denmark	FN	700	1200	NA	Duroc, Danish Landrace, Danish Yorkshire	30	50	Batch 1: 149 Batch 2: 151	Batch 1: 153 Batch 2: 155

¹ FN: Farrow to nursery; FF: Farrow to finish

BHZP: German National Hybrid Breeding Program; NA: Not available; n: Number of enrolled pigs; No.: Approximate number; ~days: Approximate number of days

Experimental timeline in the 6 tested nursery sites GA, GB, GC, GD, GE and DA. Vaccination, represented as an asterisk, was performed at approximately 21 days of age in all sites (study day 0). The white triangle and the black triangle represent the peri-vaccination weight and the exit weight, respectively. The grey vertical rectangle is the weaning. The scale is the approximate age of the pigs. Numbers in parentheses are the study day.

Preparation And Administration Of Vaccine And Control Products

Water supply was withheld for approximately two hours prior to the planned bowl vaccination in GA to stimulate drinking of the vaccine/control suspension. The live *E. coli* bivalent F4/F18 vaccine was prepared as per the Summary Product Characteristics (SPC) recommendations for vaccination of each pig with a single oral dose via drinking water systems and consumed within 4 hours after preparation. Briefly, the vials were reconstituted and then diluted with a 5-g/L skimmed milk solution. The skimmed milk solution was used as a stabilizer and for blinding purpose. The required volume per pen was calculated and prepared according to the results of a 4-hour consumption pretest and to the average piglet live body weight. Each dose was diluted to get 25 mL of the vaccine suspension per kg live weight. The same amount of the skimmed milk powder solution was administered to the control piglets, based on their body weight. Animals of GB and GC were vaccinated using automatic drenchers administering a single oral dose of 2 mL per pig after being reconstituted and diluted according to the SPC recommendations using a skimmed milk powder solution (5 g/l). Control pigs received 2 mL orally of the skimmed milk powder solution only. Piglet vaccination at GD, GE and DA was done pre-weaning using bowls. The live *E. coli* bivalent F4/F18 vaccine and the control treatment were prepared as described for GA.

Health Observations

Health observations were performed daily by the farm personnel. The severity of diarrhea was scored as 1 (mild), 2 (moderate) and 3 (severe).

Weight, Daily Weight Gain And Feed Conversion Rate

Pigs were individually weighted at study day -1 ± 1 (GA: -2, GB: -2, GC: 0, GD: -1, GE: 0, DA: -1) and then at the end of the nursery. Average weight gain (WG) and average daily weight gain (DWG) were compared between groups from days -1 ± 1 to end of nursery.

The feed intake (FI) was evaluated on GA (individual pen level) and GC (two pens combined) using data available electronically by the automated feeding system. The feed conversion rate (FCR) was calculated by dividing the average daily FI by the arithmetic mean of the average DWG of animals within the respective experimental unit (individual pen for GA and combined pens for GC). No FI measurement was available for the other sites.

Statistics And Analysis Populations

The observation unit for WG and DWG was the individual animal. For each study, these parameters were analyzed for pigs that completed the study, first for each individual site and then for all sites commingled. The observation unit for FI and FCR were the individual pen for GA and combined two pens for GC based on the technical equipment of the farms. Groups were compared using the Wilcoxon Mann-Whitney test (SAS® version 9.3). A 5% level of significance ($P < 0.05$ for two-sided tests) was used to assess statistical differences.

Results

General Health, Mortality And Reason For Non-completion

Study 1: Mild transient diarrhea was observed in two pens of the GA control group for a total of 67 (18.2%) pigs. These were not treated and recovered within 24 hours. In GC only 8 animals (1.1%) showed mild diarrhea. On the other hand, a major outbreak of diarrhea caused by a combination of *Salmonella* Typhimurium and *E. coli* (F18:Sta:Stx2e) occurred in GB from 28 days post-vaccination, which required treatment of all animals of both groups with colistin.

Overall, 61 vaccinated (5.5%) and 40 control (3.5%) animals did not complete the study 1. Among these, 28 (2.5%) vaccinated and 20 (1.8%) control pigs were removed due to protocol non-compliance. Twenty-one (1.9%) vaccinated pigs (GA: 7, GB: 8, GC: 6) and 17 (1.5%) control pigs (GA: 5, GB: 8, GC: 4) were found dead or were euthanized for ethical reasons. Clinical signs and post-mortem analyses associated the death to various causes, each observed in only few animals, such as pneumonia, lameness, crushed piglets, diarrhea, meningitis, and arthritis.

Study 2: Mild diarrhea was observed for a few pigs or in a few pens in all 3 sites. An outbreak of respiratory disease (influenza with bacterial infections) in GD resulted in the treatment of all animals about one-week post-vaccination with tiamulin hydrogen fumarate, tulathromycin and metamizole. *E. coli* edema disease was clinically observed and confirmed with isolation of an F18:Stx2e *E. coli* strain in samples collected at farm GE. However, at this farm no further batch was included due to a severe outbreak of porcine parvovirus after completion of the first batch. Finally, no other health problem than mild diarrhea for a few pigs was observed in DA.

Overall, a total of 20 (2.1%) vaccinated (GD: 4, GE: 8, DA: 8) and 21 (2.5%) control (GD: 8, GE: 6, DA: 7) animals did not complete the study 2. All these pigs were found dead or euthanized for ethical reasons. Like the first study, the death was associated to various causes observed each in only few animals such as pneumonia, lameness, crushed piglet, diarrhea, meningitis and arthritis.

There were no adverse events observed in any of the sites related to the study product.

Weight Gain And Daily Weight Gain

Body weight and WG are presented in Table 2. During the nursery period, WG of vaccinated pigs was 0.80 kg to 1.24 kg significantly higher ($P < 0.05$) than the WG of the control pigs for GA, GC, and GD and 0.69 kg higher for DA ($P = 0.051$). This difference resulted in heavier vaccinated pigs at the end of the nursery than control pigs, with differences ranging in average from 0.45 kg to 1.24 kg per animal. For sites GB and GE, occurrence of non-ETEC enteric infections described above affected the outcome of the studies; thus, no statistically significant difference between vaccinated and control pigs in WG being observed. When combining the data of all sites, WG was significantly higher for vaccinated pigs than control pigs: in average 0.56 kg ($P = 0.038$) and 0.88 kg ($p < 0.001$) more for the first and second study, respectively.

Similar results were observed for DWG, which was significantly higher for vaccinated pigs compared to control pigs for GA (by 16 g), GC (by 20 g) and GD (by 25 g) and numerically higher, though close to significance ($P = 0.051$), for DA (by 14 g) (Table 3 and Fig. 2). When data of the respective studies are combined, a significant difference of 13 g (study 1; $P = 0.028$) and 17 g (study 2; $P = 0.001$) were observed in favor of vaccinated pigs. DWG was not significantly different for GB and GE where concomitant non-ETEC enteric outbreaks occurred.

Table 2
Body weight and weight gain (WG) for the nursery period

Study	Site	n	Start weight (kg)		End weight (kg)		WG (kg)		P-value ²	
			Vaccinated pigs ¹	Control pigs ¹	Vaccinated pigs	Control pigs	Vaccinated pigs	Control pigs		
First	GA	371	369	5.30	5.30	28.99	28.19	23.69	22.89	0.033
	GB	319	342	4.29	4.59	17.71	18.48	13.42	13.89	0.161
	GC	358	382	5.28	5.70	22.69	22.24	17.41	16.54	0.035
	All sites	1048	1093	4.99	5.22	23.40	23.07	18.42	17.86	0.038
Second	GD	431	434	5.36	5.37	24.13	22.89	18.77	17.53	< 0.001
	GE	89	87	5.97	5.99	32.29	32.80	26.32	26.81	0.840
	DA	292	301	4.84	5.08	25.78	25.33	20.94	20.25	0.051
	All sites	812	822	5.24	5.33	25.62	24.83	20.38	19.50	< 0.001

¹ Vaccinated pigs received one oral dose of Coliprotec F4/F18 vaccine prepared in a 5 g/L skimmed milk solution either via bowl or by drenching at 20 ± 1 days of age; control pigs received the skimmed milk solution only, via the same route.

² P-value based on the Wilcoxon rank sum statistic

n: Number of pigs that completed the study; Kg: kilogram

Table 3
Average daily weight gain (DWG) for the nursery period

Study	Site	n		DWG (g)		P-value ²
		Vaccinated pigs ¹	Control pigs ¹	Vaccinated pigs	Control pigs	
First	GA	371	369	474	458	0.034
	GB	319	342	244	253	0.166
	GC	358	382	414	394	0.036
	All sites	1048	1093	384	371	0.028
Second	GD	431	434	383	358	< 0.001
	GE	89	87	337	344	0.840
	DA	292	301	411	397	0.051
	All sites	812	822	388	371	0.001

¹ Vaccinated pigs received one oral dose of Coliprotec F4/F18 prepared in a 5 g/L skimmed milk solution either in bowl or by drenching at 20 ± 1 days of age; control pigs received the skimmed milk solution only, via the same route.

² P-value based on the Wilcoxon rank sum statistic

n: Number of pigs that completed the study; g: gram

Average Daily Feed Intake And Feed Conversion Rate

No statistically difference was observed for the FI and FCR for GA, GC and when data of both sites are combined, though the FCR was numerically lower for vaccinated pigs than for control pigs for the analyses of the different populations.

Table 4
Average daily feed intake (FI) and feed conversion rate (FCR) for the nursery period of the study sites GA and GC

Site	n	FI (g)		FCR		P-value ²
		Vaccinated pigs ¹	Control pigs ¹	Vaccinated pigs	Control pigs	
GA	12	11	703	705	1.48	0.131
GB	5	4	673	672	1.63	0.906
GC	17	15	694	695	1.53	0.141

¹ Vaccinated pigs received one oral dose of Coliprotec F4/F18 prepared in a 5 g/L skimmed milk solution either in bowl or by drenching at 20 ± 1 days of age; control pigs received the skimmed milk solution only, via the same route.

² P-value based on the Wilcoxon rank sum statistic

n: Number of pen (GA) or combined 2 pens (GC)

Discussion

The results of this study show a positive effect of vaccinating pigs with an oral live *E. coli* bivalent F4/F18 vaccine in farms facing negligible clinical signs related to *E. coli* infections after weaning. The vaccination resulted in significantly higher weight gain

compared to non-vaccinated pigs. Results from reported field trials and laboratory challenge assays confirmed that vaccination with such PWD oral live *E. coli* vaccines improved the health of pigs and reduced ETEC intestinal colonization, ETEC fecal shedding, use of antibiotics and weight loss due to either F4-ETEC and/or F18-ETEC (Meymerit et al., 2019; Gibellini and Perro, 2019; Fily et al., 2019; Vangroenwegue and Bonny, 2018; Vangroenwegue and De Braekeleer, 2018; Vangroenwegue et al., 2018; Piqué et al., 2018; Vangroenwegue, 2017; Sánchez Uribe et al., 2017; Nadeau et al., 2017; Gibellini et al., 2017; Fairbrother et al., 2017; Nadeau et al., 2016). These previous studies were performed in proven cases of PWD associated with significant clinical outcomes such as death, diarrhea and weight loss. However, there was a need to assess the impact of vaccination of herds where the load of pathogenic *E. coli* is low resulting in mild clinical signs or not clinically noticeable by the producer or the veterinarian. The latter can be referred to as subclinical PWD.

Four out of the six tested farms (GA and GC of the first study; GD and DA of the second study) had no major outbreak of concomitant enteric diseases. In those farms, ETEC problems were mild or not clinically noticeable during the study as demonstrated by the small number of dead or euthanized pigs, the non-ETEC confirmation in samples taken from death/euthanasia of these animals and finally, the small number of animals with marked diarrhea. Mild transient diarrhea was observed in 19% of the GA control pigs. However, no *E. coli* diagnosis was performed since the diarrhea was very mild and pigs recovered in about 24 hours without treatment. The fact that only control pigs suffered from this mild transient diarrhea suggests a protective effect of the vaccination against F4- and/or F18-ETEC strains in the herd, though other causes cannot be excluded. On this farm, vaccination resulted to 0.8 kg improved nursery WG for vaccinated piglets compared to control animals. Regarding the site GC, diagnostics done in PWD cases in weaned batches prior to the study identified F4:STa:STb as the cause and confirmed that the site was at high risk of ETEC infections. The same site experienced similar diarrhea episodes after the study, also caused by the F4:STa:STb strain, but in addition an F18:STa:STb strain. Interestingly, only 8 animals had mild diarrhea and no pathogenic *E. coli* strains were detected during the study, confirming the absence of a PWD outbreak on this farm while vaccination study was carried out. It is reported that Coliprotec® F4/F18 reduces the fecal shedding of F4-ETEC and F18-ETEC by infected pigs (Nadeau et al., 2017). Therefore, vaccination of half of the litters may have reduced the ETEC bacterial load of the herd to a level limiting the occurrence of ETEC-related clinical signs after weaning, but without eradicating the infection in the control group. This is supported by the fact that the vaccination resulted in higher nursery WG of 0.87 kg and DWG of 20 g/day.

The Danish site (DA) showed the lowest risk for ETEC diseases as no recent ETEC problem had been diagnosed before the study started. Furthermore, no diarrhea was observed during the study except for very few animals with transient mild diarrhea. Despite this very low ETEC infection pressure, WG during the nursery period was statistically significantly higher for the vaccinated pigs compared to the control pigs; though it was the lowest difference (0.69 kg) observed amongst the four farms without concomitant enteric outbreaks.

The site GD was associated with the most important reduction of weight loss by vaccination: vaccinated pigs gained 1.24 kg more weight ($P < 0.001$) than control pigs by the end of the nursery period. This site faced an influenza episode complicated with bacterial infections that led to the treatment of all pigs with tiamulin, tulathromycin and metamizole. It is possible that the stress caused by the viral infection may have increased the differences in WG between vaccinated and control pigs.

For sites GB and GE, where concomitant clinical enteric diseases have been diagnosed, no difference in weight gain between vaccinated and control pigs was observed. These two farms had overall the lowest nursery DWG amongst the tested farms reflecting the negative impact of the clinical outbreaks of enteric diseases on the health of the pigs, whether the pigs had been vaccinated with the live *E. coli* oral bivalent vaccine or not. Since the protective effect of the oral live *E. coli* bivalent F4/F18 vaccine is related to specific anti-F4 and anti-F18 secreted IgM and IgA (Nadeau et al., 2017), it is expected to have no preventive effect on concomitant enteric diseases such as *Salmonella* or parvovirus. More studies are needed to determine the impact of concomitant systemic and enteric outbreaks on the effects of the live *E. coli* oral bivalent vaccine when administered to piglets subjected to subclinical cases of PWD.

Diarrhea caused by ETEC is a result of multiple factors and is clinically observed when the amount of fluid from the small intestines exceeds the colonic capacity for fluid reabsorption. To induce noticeable clinical signs, ETEC strains must attach to specific receptors onto the jejunal and ileal epithelium brush borders and then proliferate while secreting enterotoxins leading to reduction of intestinal absorption and fluid movement into the intestinal lumen (Fairbrother and Nadeau, 2019; Nagy and Fekete,

2005). The most important *E. coli* enterotoxins in pigs are STa, STb, LT and EAST1 (Fairbrother and Nadeau, 2019). To induce noticeable diarrhea in nursery pigs, large amount of ETEC (at least 10^9 to 10^{10}) colonizing and proliferating in the intestines are required (Sun and Kim, 2017). The degree of colonization and multiplication of ETEC in the intestine determine whether the infection results in clinical signs, including diarrhea. On the other hand, the absence of noticeable diarrhea in a herd does not exclude a negative impact of the ETEC infection on the pig gut health and performance. Challenging healthy susceptible piglets with a pathogenic strain of ETEC may induce severe diarrhea, but it can also induce a subclinical form of PWD (Opapeju et al., 2009). Subclinical infections without noticeable diarrhea or mortality but with marked intestinal colonization following ETEC exposure are reported in the literature for porcine challenge models (Pollock et al., 2019). It is also reported that ETEC colonization at a subclinical level after weaning can negatively affect the growth performance of pigs (Athanasiadou et al., 2010; Hampson, 1994).

E. coli enterotoxins harbor functions beyond fluid and electrolyte hypersecretion that can impair the gut health. As examples, STb is able to alter the tight junction complexes that increases the permeability of the intestinal epithelium and might modulate innate immune responses, while STa enhances the secretion of pro-inflammatory cytokines and chemokines in the small intestinal lumen (Wang et al., 2019). ETEC infection is also associated with an important microbiota change in the distal small intestines but not in the feces (Pollock et al., 2019). Although diarrhea may not be triggered by an ETEC infection, the enterotoxin functions, other than hypersecretion, may affect the gut health in the jejunum and the ileum, being the major colonization sites for porcine F4-ETEC and F18-ETEC (Fairbrother and Nadeau, 2019) and where a high uptake of nutrients occurs. The impact of subclinical ETEC infections may be exacerbated following weaning when a decrease of digestive and adsorptive capacities of the intestines due to villous atrophy and crypt hyperplasia are observed (Thompson and Friendship, 2019).

Knowing that oral live *E. coli* swine vaccines significantly reduce the ETEC load in the intestine and shedding in the feces (Fairbrother et al., 2017; Nadeau et al., 2017), the higher weight gain observed for vaccinated pigs may be related to a control of this potential impairment of the gut health by the subclinical ETEC infection. However, a direct impact of such live *E. coli* oral vaccines on gut health cannot be excluded as these vaccine strains colonize the intestines at the peri-weaning moment; when the establishment of the post-weaning intestinal flora is critical. Accordingly, it has been reported that the oral administration of Coliprotec® live bivalent F4/F18 *E. coli* vaccine just before weaning enables the stabilization of the intestinal microbiota after weaning with the proliferation of short-chain fatty acid (SCFA)-producing bacteria, such as *Prevotella* sp., known to be beneficial to gut health (Luise et al., 2020). Interestingly, the authors also reported a higher nursery average daily weight gain for the vaccinated pigs than for the non-vaccinated pigs.

The existence of subclinical ETEC infection in pigs is not well described in the literature, though reported by few research groups (Pollock et al., 2019; Athanasiadou et al., 2010; Opapeju et al., 2009; Hampson, 1994). One reason for this is the difficulty to detect ETEC in healthy looking pigs. Confirmation of the presence of ETEC in pigs is based on the bacteriological examination of fecal or intestinal content samples on agar plates, selection of isolated colonies and identification by PCR of genes for the fimbriae and the toxins (Fairbrother and Nadeau, 2019; Luppi et al., 2017). This diagnostic approach is sensitive enough for clinical cases of ETEC for which it is possible to select few piglets in the acute phase of diarrhea; when the ETEC fecal load is high enough to be distinguished on the agar plates from the normal *E. coli* microbiota. However, in mild or subclinical cases of ETEC infections, it is not possible to select few pigs that are more likely to heavily shed ETEC as they all look healthy. Furthermore, the fact that a weaning batch is composed of either susceptible or resistant pigs for ETEC infection makes the sampling more difficult; the susceptibility being driven by the presence of specific receptors for F4 and F18 fimbriae onto the intestinal epithelium (Luise et al., 2019). The diagnostic approach used for acute ETEC infection is therefore less appropriate for the detection of ETEC in subclinical cases of PWD. It would necessitate high number of sampled pigs and the detection of genes by a fairly sensitive method. Though achievable for study purposes, this approach would be cost-prohibitive for routine diagnostic. One approach may be to sample many pigs on a farm using rectal swabs and then to pool the samples in batches to reduce the number of analyses for the detection of the genes by real-time PCR directly in suspended pooled samples. The validation of such an approach would be required to confirm its suitability to detect subclinical cases of PWD.

The combination of colistin and therapeutic levels of zinc oxide was a widely used metaphylactic practice across the European Union and in many other regions of the world to control PWD. However, several measures to reduce the use of colistin in food-producing animals have been recently adopted by EMA following the identification of a transferable MCR-1 resistance mechanism

(EMA, 2016). Colistin is now classified as a Category 2 antibiotics, which means it should be reserved for treating infections in pigs for which no effective alternative treatments exist. In this sense, EU Member States should reduce the use of colistin in pigs at pre-established thresholds without compensation in the use of other categories of antimicrobials. The metaphylactic use of 2400 to 3000 ppm of zinc in the feed is known to reduce diarrhea caused by *E. coli*, mortality and improve growth of pigs (Luppi, 2017). However, veterinary medicinal products containing zinc oxide will no longer be permitted in the EU from June 2022, following a negative benefit/risk conclusion of EMA's CVMP evaluating the current status of safety and effectiveness (EMA 2017). The already initiated discontinuation of this metaphylactic approach in many European countries opens the search for effective sustainable strategies to control ETEC infections in pig farms. This transitional period is expected to lead to major changes in overall swine gut health, mainly resulting from the trial-and-error approach that could be considered to find alternatives to compensate the economic losses incurred by the withdrawal of the previously used antibiotic products.

Our parallel group blinded studies revealed that mild or subclinical cases of F4- and F18-ETEC infection in pig nurseries can have a negative impact on the growth performance and that pigs orally vaccinated with an oral live *E. coli* bivalent F4/F18 vaccine showed a higher weight gain from about half a kilogram to more than one kilogram per pig on average from the weaning to the end of the nursery period. It is noteworthy that pigs vaccinated peri-weaning maintained a higher weight gain throughout the entire nursery phase in the case of subclinical/mild ETEC infection, likewise described by others in farm trials with confirmed clinical cases of PWD (Meymerit et al., 2019; Gibellini and Perro, 2019; Fily et al., 2019; Vangroenwegue and De Braekeleer, 2018; Vangroenwegue et al., 2018; Piqué et al., 2018). Furthermore, though not statistically different, the FCR was numerically lower for vaccinated pigs than control pigs in both farms where the feed intake was evaluated. The study design limited, however, the assessment of the FCR, particularly due to the low number of experimental units tested and further studies specifically designed for that purpose are needed to make a clear conclusion of the impact of vaccination with this oral live *E. coli* bivalent vaccine for this parameter.

Conclusions

These current studies demonstrate that, even in the absence of convincing clinical signs of PWD, ETEC infections limit the nursery piglet optimal growth performance and that these effects can be mitigated by vaccination with an oral live *E. coli* bivalent F4/F18 vaccine. These findings add to studies proving the effect of the vaccine in clinical cases of PWD which clearly demonstrated the efficacy of the oral live bivalent *E. coli* F4/F18 vaccine not only in reducing clinical signs of PWD but also in reducing the negative impact of an ETEC infection on pig growth performance. Further studies are needed to evaluate the importance and the impact of these subclinical ETEC infections in the swine industry and to characterize the effect and the role of the oral live *E. coli* bivalent vaccine in these cases.

Abbreviations

DWG
Daily weight gain
EAST1
Enterotoxigenic heat-stable toxin 1
ETEC
Enterotoxigenic *Escherichia coli*
F4
Fimbriae F4
F18
Fimbriae F18
FCR
Feed conversion ratio
FI
Feed intake
LT

Heat-labile toxin
PWD
Post-weaning diarrhea
SPC
Summary of Product Characteristics
STa
Heat-stable toxin a
STb
Heat-stable toxin b
Stx2e
Shigatoxin 2e
WG
Weight gain

Declarations

Ethics approval and consent to participate

- Field trials performed with a veterinary medicinal product approved for use in swine. No additional ethical approval needed. Owner consent to participate was obtained for all owners using an Owner informed consent form following Good Clinical Practices guidelines.

Consent for publication

- Not applicable

Availability of data and materials

- Please contact the author for requests.

Competing interests

- This project was sponsored by Prevtect Microbia. The sponsor made substantial contributions to the study design, conduct of the study, collection, analysis, and interpretation of the data, and the writing of the manuscript. The sponsor of this research is the market authorization holder of the Coliprotec F4/F18 vaccine. EN and DT were, at the time of the study, employees of the sponsor. EN is co-inventor of Coliprotec F4/F18. KH was compensated by the study sponsor for his participation as the principal investigator and study principal monitor while LMN acted as study monitor. EN and DT are current employee of the actual owner and the distributor of Coliprotec F4/F18.

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Authors' contributions

- All authors contributed to the design of the study and interpretation of results. EN, DT and KH managed and supervised the studies. KH and LMN monitored the studies. All authors read and approved the final manuscript.

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Figures

Figure 1
Experimental timeline in the 6 tested nursery sites

Figure 2

Average daily weight gain (DWG) for the nursery period of the first (a) and second (b) study Comparison of the daily weight gain (DWG) over the total study period (the entire nursery period; study day -1 $\pm$ 1 to the end of the nursery) between pigs vaccinated with an oral live *E. coli* bivalent F4/F18 vaccine and non-vaccinated control pigs. For the first study (a) and the second study (b) results are presented for individual study site and for all study sites combined. Data are reported as the mean and error bars represent the standard error. * Daily weight gain of the vaccinated pigs significantly higher than that of the control pigs ($P < 0.05$).

** $P = 0.051$