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Administration of a novel plant extract product via drinking water to post-weaning piglets: effects on performance and gut health

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The present study evaluated the effects of a novel plant extract (PE) product (Grazix^{TM}) on the performance and gut health of weaned piglets challenged with Escherichia coli. *The PE was a standardised mixture of green tea leaves (*Camellia sinensis*) and pomegranate fruit (*Punica granatum*) obtained by using the LiveXtract^{TM} process. A total of 144 piglets were weaned at 24 days and allocated to 8 for a 35-day experiment with a 2 × 2 × 2 factorial design comparing different treatments (water without product (CT) or 8 μl/kg per day PE in drinking water (PE)), feeding regimens (*ad libitum *(AD) or restricted (RE)) and oral* E. coli *challenges on day 9 (sham (−) or infected (+)). There were six pens per group with three piglets per pen. On day 35, 24 of the RE feeding piglets were slaughtered. It was found that PE supplementation increased the average daily gain (ADG) from day 28 to day 35 (*$P = 0.03$*) and increased the gain to feed ratio (G : F) from day 7 to day 14 (*$P = 0.02$*). RE feeding led to lower feed intake in piglets during the 1st week (*$P < 0.01$*), 2nd week (*$P = 0.06$*), 3rd week (*$P = 0.05$*), and throughout the course of the overall study period (*$P = 0.05$*).* E. coli *challenge decreased the ADG and G : F ratio from day 7 to day 14 (*$P = 0.08$ *and* < 0.01*, respectively) and increased the faecal score (higher values indicate more severe diarrhoea) on days 14, 21, 28 and 35 (*$P < 0.01$*). PE supplementation decreased the faecal score in the challenged piglets during the 1st week post-challenge (*$P < 0.01$*).* E. coli *challenge increased the faecal* E. coli *level on day 14 (*$P = 0.03$*) and increased the* Enterobacteriaceae *level on day 35 (*$P < 0.01$*). Reduced faecal* E. coli *was observed on days 14 and 35 (*$P = 0.05$ *and* 0.02*, respectively), and reduced* Enterobacteriaceae *(*$P < 0.01$*) was found on day 35 in the PE animals. RE feeding increased the faecal* Lactobacillus, Enterobacteriaceae *and* E. coli *levels on day 35 (*$P = 0.02$*,* < 0.01 *and* < 0.01*, respectively). These results suggest that PE supplementation may improve the gut health status of post-weaning piglets and counteract some of the negative effects that occur when piglets are challenged with* E. coli.

Keywords: *Escherichia coli*, gut health, performance, piglet, plant extract

Implications

This study investigated the effects of a novel plant extract product that was added to the drinking water for weaned piglets. Different feeding regimens were tested, and the effects of the extract on growth performance, gut health and protection against *Escherichia coli* challenge were observed. The results showed that the plant extract may represent a useful additive for improving gut health and microbial ecology and reducing the severity of an *E. coli* challenge. The results may have a significant impact on nutritional management in conventional farms. Use of the plant extract could enable piglets to better withstand the infections that are often associated with weaning.

Introduction

Phytobiotics is a term used to describe plant-derived natural bioactive compounds that promote livestock health and well-being and improve livestock growth and production efficiency (Wu and Wu, 2012). Although the mechanisms of phytobiotics are not entirely understood, their benefits to the overall health of animals have been noted. Phytobiotics represent a source of various chemical and bioactive compounds, such as terpenes, phenols, glycosides, saccharides, aldehydes, esters and alcohols. When plant extracts (PEs) have been used in swine nutrition, both improvements and reductions in productivity have been observed; however, this

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has also been the case for common growth promoters (Manzanilla *et al*., 2006; Nofrarías *et al*., 2006; Jin *et al*., 2008). The beneficial effects of phytobiotics may be explained by the activation of feed intake, the secretion of digestive enzymes, immune stimulation, intestinal microflora modulation, anti-bacterial effects and anti-inflammatory properties (Ultee *et al*., 2002; Windisch *et al*., 2008).

Increased susceptibility to enterotoxigenic *Escherichia coli* infections and acute diarrhoea are common problems in newly weaned animals. Experimental models for *E. coli* K88 challenge have been used to evaluate the role of feed additives for weaning pigs in modulating the gastrointestinal microbial response (Bhandari *et al*., 2008; Kiarie *et al*., 2011). Feed restriction, as part of farm strategy, has been applied in post-weaning piglets to decrease the proliferation of bacteria such as haemolytic *E. coli* and to reduce the incidence of diarrhoea, and it also has been associated with a transitory decrease in growth compared with piglets fed *ad libitum* (AD) (Pastorelli *et al*., 2012). Thus, the current study was conducted to investigate the effects of a novel PE product on the growth performance and gut health (microbial counts, intestinal morphology and enzyme activities) of weaned piglets undergoing an experimental *E. coli* challenge and feed restriction.

Material and methods

The experimental protocol was reviewed and approved by the Animal Care and Use Committee of the University of Milan (Protocol No. Gra.Piglet.09.10).

Animals and treatments

The experiment was carried out at the facility for Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy). At weaning (24 days), a total of 144 crossbred (Stambo HBI × Dalland) piglets (6.50 ± 0.35 kg) of the same age and litter origin were randomly assigned to treatment groups. Animals were housed in two identical rooms, equipped with 24 pens each, in an environmentally regulated, isolated stable. A combination of daylight and artificial light was used. Ventilation was achieved by using variable-speed fans. The starting temperature of 28ºC was adjusted weekly to reach a final temperature of 24ºC. Piglets were housed in pens with a slatted floor (3 piglets/pen, 1.20 × 1.00 m). Each pen was equipped with two standard nursery pig bite-style nipple drinkers or stainless steel nursery push-lever bowl drinkers and a self-feeder. Piglets were raised for 35 days in eight different groups with a 2 × 2 × 2 factorial design comparing different treatments (water without product (CT) or 8 μl/kg per day PE in the drinking water (PE)), feeding regimens (AD or restricted (RE)) and oral *E. coli* challenges (sham (−) or infected (+)). There were six pens per treatment group, and the different combinatorial treatments are designated as follows: (1) CTAD −; (2) CTAD +; (3) CTRE −; (4) CTRE +; (5) PEAD −; (6) PEAD +; (7) PERE −; and (8) PERE +.

Table 1 *Composition and calculated nutrient content of the basal diet*[1]

Ingredient (g/kg diet)	
Wheat	593.4
Whey powder	95.0
Soy protein concentrate	70.0
Herring meal	55.0
Wheat bran	50.0
Soybean meal	47.8
Soybean oil	25.0
Dextrose monohydrate	22.0
Dicalcium phosphate	12.6
Pig lard	8.0
Lysine HCl 78	5.0
Calcium carbonate	4.7
L-Threonine	4.0
DL-Methionine	2.5
Vitamin premix[2]	2.5
Salt	1.5
L-Tryptophan	1.0
Calculated nutrients (g/kg diet)	
Dry matter	896.3
CP	198.5
Ether extract	50.7
Crude fibre	23.8
NDF	98.9
Ash	58.7
Total lysine	15.3
Total sulfur amino acid	9.4
Calcium	8.8
Phosphorus	7.5
Sodium	2.2
Threonine	11.4
Tryptophan	3.3
Metabolizable energy (Mcal/kg)	3.53

[1]Diets were not supplemented with antibiotics.
[2]Vitamin-mineral premix contents per kg final feed: Vitamin A: 10 500 IU; Vitamin D_3: 2500 IU; Vitamin E: 15 mg; Vitamin B_1: 1.5 mg; Vitamin B_2: 3.8 mg; Vitamin B_{12}: 0.025 mg; Vitamin B_6: 1.6 mg; calcium pantotenate: 12 mg; nicotinic acid: 15 mg; biotin: 0.15 mg; folic acid: 0.5 mg; Vitamin K_3: 3 mg; Fe: 100 mg; Cu: 6 mg; Co: 0.75 mg; Zn: 150 mg; Mn: 65 mg; I: 0.75 mg; Se: 0.4 mg; ethoxyquin: 150 mg.

On day 9 of the trial, the piglets housed in one of the two rooms were orally injected with 4 ml of a solution containing 10^9 cfu of *E. coli* 0149: F4 (K88)-positive strain. The K88-positive strain, isolated from pigs with colibacillosis, also expressed heat-labile and heat-stable B toxins, and further prepared as described by Bosi *et al.* (2004).

The PE product (Grazix™, LiveLeaf Inc., San Carlos, CA, USA) is an oral supplement produced by using the LiveXtract™ process on green tea leaves (*Camellia sinensis*) and pomegranate rinds (*Punica granatum*). The PE product was administered from 2000 to 0800 h through the drinking water. A graduated tank filled with treated water was linked to the nursery push-lever bowl drinker in each pen. The PE product was diluted with water daily and manually supplemented in the water tank to maintain the correct dosage according to the total animal weight per pen (8 μl/kg per day). This adjustment was carried out at 2000 h, and the

provision of water was managed to ensure that the piglets consumed all of the water. Following the *E. coli* challenge (day 9), PE supplementation was increased; the *E. coli*-challenged piglets received 200 μl/kg per day of the PE product on days 8, 9 and 12, and they received 400 μl/kg per day on days 10 and 11. The diet was formulated to be iso-nutritive, exceeding the protein requirement recommended by NRC (1998) for pigs (Table 1), and it did not include any antibiotic growth promoters or antibiotic growth promoter alternatives. In the RE feeding groups, piglets were allowed access to food from 0800 to 2000 h. Feeding troughs for the RE groups were removed at 2000 h, weighed and stored. Each morning, the feeding troughs were filled, weighed and placed in the pens at 0800 h.

Experimental observations and measurements

All piglets were weighed at weaning (day 0) and subsequently every week until the end of the trial. Piglet feed consumption was measured on a daily basis in the RE regimen groups, and it was measured weekly in the AD regimen groups. The average daily gain (ADG), average daily feed intake (ADFI) and gain to feed ratio values (G : F) were calculated for each pen. Water consumption was measured daily as the amount moved from the graduated tank to the nursery push-lever bowl drinker. The occurrence and severity of diarrhoea were monitored weekly. After the *E. coli* challenge, the faecal score daily monitored daily for 1 week in the challenged animals. The severity of diarrhoea was characterised by using a 5-point faecal consistency scoring system: 1 = hard, dry pellet; 2 = firm, formed stool; 3 = soft, moist stool that retains shape; 4 = soft, unformed stool; 5 = watery liquid that can be poured. Liquid consistency (score 4 to 5) was considered indicative of diarrhoea. Pooled faecal samples from each pen (~20 g), were collected on days 0, 14 and 35, placed in small sterile containers, and immediately sent to the laboratory for microbiological analysis. One gram of the fresh sample was diluted with 99 ml sterile physiological NaCl solution. Following homogenisation on a vortex, 1 ml of the suspension was mixed with 9 ml NaCl solution. Serial dilutions (1 : 10) for culturing were prepared down to 10^{-9}. From each diluted sample, 0.1 ml was plated on the appropriate medium for enumeration of microbial populations. Three replicates were carried out for each sample. Plates for the enumeration of *Enterobacteriaceae* (using Violet-Red Bile Dextrose agar) were incubated aerobically at 37°C for 24 h. *E. coli* was grown in tryptic soy agar at 37°C. *Lactobacillus* faecal content was determined using MRS agar (*Lactobacillus agar*) with an incubation time of 72 h at 37°C (10% CO_2), and the *Clostridia* procedure used an incubation time of 48 h at 37°C with tryptose sulphite cycloserine agar. The microbial counts were expressed as $\log_{10}$ cfu/g.

At the end of the trial, 24 animals from the RE groups (3 piglets per treatment per room) were selected as the most representative of pen performance in terms of weight gain and health, and these animals were slaughtered. The animals were stunned electrically and bled after ~16 h of starvation. Immediately after slaughter, the gastrointestinal tract was removed from each animal, and the distal ileum (2 cm before its opening into the caecum) was collected and promptly fixed in neutral buffered formalin for 24 h at 4°C. The specimens were then dehydrated in a graded ethanol series, cleared with xylene and embedded in paraffin. After dewaxing and rehydration, microtome sections (4 μm thick) were stained with haematoxylin and eosin and examined to either assess the ileum micro-anatomical structure or perform histometry. Histometric assays considered the following parameters for each section: villus height (V) (five villi measured per section), crypt depth (C) (five crypts measured per section), the villus height to crypt depth ratio (V : C ratio), number of lymphatic follicles (counted in 3 fields per section at 400× and expressed as n/mm^2 of mucosa) and the area of the lymphatic follicles and their compartments (cortex, medulla, corona; 5 follicles per section). Other ileum sections were processed by immunohistochemical analysis to identify mucosal macrophages with a macrophage monoclonal antibody (1 : 400, ab22506, Abcam) after antigen retrieval with K-protease (20 μg/ml of buffer solution). For each section, the number of immuno-positive mucosal cells was counted in eight fields at 400× and subsequently expressed as n/mm^2 of mucosa.

The ileal mucosa was also used to evaluate myeloperoxidase (MPO), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) by using commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Three 5 cm segments from the ileum were collected, opened longitudinally and cleaned with phosphate buffered saline (PBS). Intestinal mucosa samples were collected by scraping the wall with a glass slide. Nine ml of 4°C PBS was added to 1 g of intestinal mucosa, followed by homogenisation. The homogenates were centrifuged (4000 × **g** for 5 min at 4°C), and supernatant fluid was used according to the manufacturer's instructions.

Statistical analysis

Data were analysed as a completely randomised block design by ANOVA, as implemented in the MIXED procedure of SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA). For growth performance, faecal scores and microbial counts, a model for a 2 × 2 × 2 factorial design was applied. The model statement included the effects of treatment (CT or PE), feeding regime (AD or RE), *E. coli* challenge (− or +), and interactions among those factors, as suggested by Song *et al.* (2012). The pen represented the experimental unit for these parameters. Faecal scores recorded for the challenged piglets within 7 days of challenge were analysed as a generalised randomised block design with repeated measurements over time, and the pen was the experimental unit. An ANOVA mixed model with a 2 × 2 factorial design was used for gut histomorphology and mucosal inflammatory parameters. The applied model included the treatment effect (CT or PE), the *E. coli* challenge effect (− or +) and a treatment × challenge interaction. The individual piglets were the experimental units for intestinal histometric and mucosal inflammatory

parameters. Treatment differences were assessed by using the least squares means with a Tukey adjustment. Treatment effects were considered significant at $P \leqslant 0.05$, whereas a trend for a treatment effect was noted for $P \leqslant 0.10$.

Results

Growth performance

The effects of PE and feeding regimen on growth performance in the *E. coli*-challenged and non-challenged piglets are shown in Table 2. The piglets that were given supplemental PE showed enhanced ADG during the last week of the trial ($P = 0.03$) and had an increased G : F ratio during the second and last weeks ($P = 0.02$ and 0.10, respectively). Reduced ADG and G : F ratio values were observed in the PE treatment from day 0 to day 7 ($P = 0.04$ and 0.06, respectively). An effect of the feeding regimen was observed on ADFI for the overall experimental trial, with a higher feed intake in the piglets that were fed AD during the 1st week ($P < 0.01$), 2nd week ($P = 0.06$) and 3rd week ($P = 0.05$), and during the overall study period ($P = 0.05$). However, the piglets with RE feeding had higher G : F ratios than those in the AD feeding group(s) from day 21 to day 28 ($P = 0.01$). The *E. coli* challenge decreased the ADG and G : F ratio during days 7 to 14, the week of the challenge ($P = 0.08$ and < 0.01, respectively), and it reduced the ADG from day 14 to day 21 and from day 0 to day 35 ($P = 0.03$ and 0.06, respectively). The treatment × regimen interactions between ADG and G : F ratio from day 0 to day 7 ($P = 0.04$ and 0.02, respectively) indicate that PE had different effects on those performance in the AD and RE regimen animals. The results indicate that G : F ratio was also affected by treatment, feeding regimen, and challenge from day 28 to day 35 and day 0 to day 35 (treatment × regimen × challenge, $P = 0.05$ and 0.02, respectively). The interactions during the last week and for the overall period can be attributed to the differences between the CTAD − (0.55 and 0.58, respectively) and PEAD − (0.75 and 0.66, respectively) groups, but no differences were found in others piglets. In addition, our results indicate that the G : F ratio tended to be affected by treatment and challenge (treatment × challenge, $P = 0.08$),

Table 2 *Effects of plant extract supplementation on growth performance of* Escherichia coli*-challenged (+) and non-challenged (−) piglets fed* ad libitum *or restricted diets*[1]

	CTAD −	CTAD +	CTRE −	CTRE +	PEAD −	PEAD +	PERE −	PERE +	s.e.m.	Response		
Days 0 to 7[2]												
Day 0 BW (kg)	6.41	6.57	6.40	6.58	6.41	6.53	6.39	6.59	0.492			
ADG (g/day)	63	76	85	67	72	68	41	47	10.8	T**	T × R**	
ADFI (g/day)	171	180	152	148	178	158	130	134	13.3	R***		
G : F	0.37	0.41	0.57	0.47	0.40	0.42	0.31	0.36	0.061	T*	T × R**	
Days 7 to 14												
ADG (g/day)	178	165	178	160	205	184	186	150	17.6	Ch*		
ADFI (g/day)	315	319	285	285	305	310	288	259	23.9	R*		
G : F	0.56	0.51	0.63	0.56	0.68	0.59	0.65	0.58	0.035	T**	Ch***	
Days 14 to 21												
ADG (g/day)	366	287	335	256	350	307	297	285	33.3	Ch**		
ADFI (g/day)	526	454	448	399	500	472	454	412	41.5	R**		
G : F	0.70	0.63	0.76	0.65	0.70	0.64	0.65	0.70	0.054			
Days 21 to 28												
ADG (g/day)	393	360	396	333	347	353	418	398	32.8			
ADFI (g/day)	688	567	588	549	606	613	616	563	48.1			
G : F	0.57	0.65	0.69	0.60	0.57	0.58	0.68	0.70	0.041	R***		
Days 28 to 35												
ADG (g/day)	466	412	394	405	582	499	455	421	43.3	T**	R**	
ADFI (g/day)	835	688	692	691	788	884	795	714	65.1		T × R × Ch*	
G : F	0.55[a]	0.60[a]	0.58[a]	0.58[a]	0.75[b]	0.59[a]	0.57[a]	0.59[a]	0.039	T*	T × Ch*	T × R × Ch**
Days 0 to 35												
ADG (g/day)	293	260	278	244	311	282	280	260	20.7	Ch*		
ADFI (g/day)	507	441	433	414	475	488	457	416	33.2	R**		
G : F	0.58[a]	0.59[ab]	0.65[b]	0.59[ab]	0.66[b]	0.58[a]	0.61[ab]	0.62[ab]	0.025	T × R × Ch**		

CT = water without supplementation; PE = 8 µl/kg per day PE in drinking water; T = treatment (CT *v.* PE); AD = *ad libitum* regimen; RE = restricted regimen; R = regimen (AD *v.* RE); +/− = presence or absence of *E. coli* challenge; ADG = average daily gain; ADFI = average daily feed intake; Ch = challenge (sham (−) *v.* infected (+)); T × R = interaction between treatment and regimen; T × Ch = interaction between treatment and challenge; R × Ch = interaction between regimen and challenge; T × R × Ch = interaction between treatment, regimen and challenge.

[a,b]Means listed in the same row with different superscripts are significantly different ($P < 0.05$).

[1]$n = 48$ (6 pens/treatment). Piglets were weaned at 24 days of age, and half of the piglets were orally challenged with *E. coli* on day 9 of the trial.

[2]Day of the trial.

*$P \leqslant 0.10$, **$P \leqslant 0.05$, ***$P \leqslant 0.01$.

and ADFI tended to be affected by treatment, regimen and challenge (treatment × regimen × challenge, $P = 0.09$) during the last week of the trial. For the PE group, piglets fed AD increased their water consumption compared with piglets with RE feeding (258 ml/piglet per day *v.* 148 ml/piglet per day; $P < 0.01$, data not shown). No difference was observed in the PE-supplemented water consumption between *E. coli*-challenged and non-challenged piglets.

Faecal score and faecal microbial population

E. coli challenge increased the faecal scores of the CT piglets, resulting in average values above four points (index of diarrhoea occurrence) up to 6 days after challenge (Figure 1). Within 1 week post-infection, there was a treatment ($P < 0.01$) and time effect ($P < 0.01$), but no treatment × time interaction for faecal score. Piglets supplemented with PE had improved faecal consistency, irrespective of their feeding regimen, compared with CT. PE supplementation decreased the faecal score of PEAD and PERE piglets compared with the CTRE group on the 2nd day post-challenge ($P = 0.05$). On the 3rd day post-challenge, the CT piglets had a higher faecal score than the PE piglets, irrespective of the feeding regimen ($P = 0.05$). In addition, diarrhoea occurrence was lower in the PE piglets than in the CT animals (26% *v.* 62%, data not shown). The effects of PE supplementation and feeding regimen on faecal score in the *E. coli*-challenged and non-challenged piglets are shown in Table 3. PE supplementation decreased the faecal score values on days 14 ($P = 0.02$), 21 ($P < 0.01$), 28 ($P < 0.01$) and 35 ($P < 0.01$). *E. coli* challenge significantly affected the faecal score on days 14 ($P < 0.01$), 21 ($P < 0.01$), 28 ($P < 0.01$) and 35 ($P < 0.01$). However, faecal score was not affected by the feeding regimen. In addition, treatment × challenge interactions were observed on days 28 ($P < 0.01$) and 35 ($P < 0.01$), indicating that PE supplementation had different effects on challenged and non-challenged piglets.

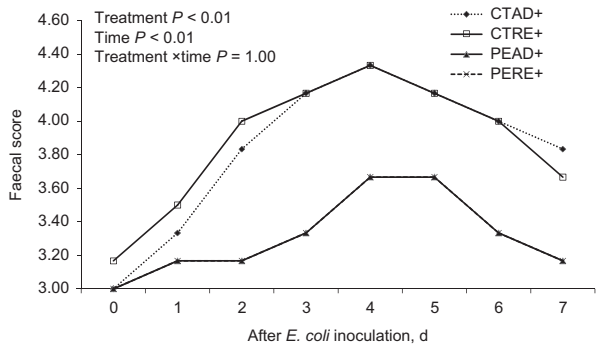

Figure 1 Effect of plant extract (PE) on faecal score in piglets with different feeding regimens 7 days after *Escherichia coli* challenge. CTAD + = no supplement in drinking water (CT), *ad libitum* regimen (AD), challenge with *E. coli* (+); CTRE + = no supplement in drinking water (CT), restricted regimen (RE), challenge with *E. coli* (+); PEAD + = 8 μl/kg per day PE in drinking water (PE), *ad libitum* regimen (AD), challenge with *E. coli* (+); PERE + = 8 μl/kg per day PE in drinking water (PE), restricted regimen (RE), challenge with *E. coli* (+). Error bars are omitted for presentation purposes. Pooled s.e.m. = 0.30. Faecal score were recorded by using a 5-point scoring system: 1 = hard; 2 = firm; 3 = soft (moist stool); 4 = soft (unformed stool); 5 = watery faeces. Diarrhoea: liquid consistency (score 4 to 5). Day 9 of the trial is shown as day 0 post-inoculation with *E. coli*.

The effects of PE and feeding regimen on faecal microbial populations in the *E. coli*-challenged and non-challenged piglets are shown in Table 4. No effect was observed on the *Clostridia* population ($P > 0.05$). PE supplementation decreased the faecal *E. coli* levels on days 14 and 35 ($P = 0.05$ and 0.02, respectively) and reduced the *Enterobacteriaceae* ($P < 0.01$) microbial population on day 35. The challenge increased the faecal *E. coli* ($P = 0.03$) and *Enterobacteriaceae* populations ($P < 0.01$) on days 14 and 35, respectively. The faecal microbial population was influenced by the feeding regimen, with an increase of faecal *Lactobacillus*, *Enterobacteriaceae* and *E. coli* populations in piglets fed the RE diet on day 35 ($P = 0.02$, <0.01 and <0.01, respectively). In addition, RE fed piglets increased faecal *Lactobacillus*, *Enterobacteriaceae* and *E. coli* population in comparison with piglets fed AD on day 35 ($P = 0.02$, <0.01

Table 3 *Effects of plant extract (PE) supplementation on the faecal scores[1] of* Escherichia coli*-challenged (+) and non-challenged (−) piglets fed* ad libitum *or restricted diets[2]*

	CTAD −	CTAD +	CTRE −	CTRE +	PEAD −	PEAD +	PERE −	PERE +	s.e.m.	Response		
Day 0[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	–			
Day 7	3.00	3.00	3.00	3.17	2.83	3.00	3.00	3.00	0.111			
Day 14	4.00	4.17	3.17	4.17	2.67	3.67	2.83	3.67	0.373	T**	Ch***	
Day 21	3.33	3.50	3.17	3.50	2.33	3.17	2.50	3.17	0.239	T***	Ch***	
Day 28	3.00	3.33	3.00	3.00	2.17	3.00	2.33	3.00	0.121	T***	Ch***	T × Ch***
Day 35	2.83	3.00	2.83	3.00	2.17	3.00	2.17	3.00	0.118	T***	Ch***	T × Ch***

CT = water without supplementation; PE = 8 μl/kg per day PE in drinking water; T = treatment (CT *v.* PE); AD = *ad libitum* regimen; RE = restricted regimen; R = regimen (AD *v.* RE); +/− = presence or absence of *E. coli* challenge; Ch = challenge (sham (−) *v.* infected (+)); T × R = interaction between treatment and regimen; T × Ch = interaction between treatment and challenge; R × Ch = interaction between regimen and challenge; T × R × Ch = interaction between treatment, regimen and challenge.

[1]Faecal scores were recorded using a 5-point scoring system: 1 = hard; 2 = firm; 3 = soft (moist stool); 4 = soft (unformed stool); 5 = watery faeces. Diarrhoea: liquid consistency (score 4 to 5).

[2]$n = 48$ (6 pens/treatment). Piglets were weaned at 24 days of age, and half of the piglets were orally challenged with *E. coli* on day 9 of the trial.

[3]Day of the trial.

**$P \leqslant 0.05$, ***$P \leqslant 0.01$.

Table 4 *Effects of plant extract (PE) supplementation on faecal microbiological counts (log_{10} cfu/g) in* Escherichia coli*-challenged (+) and non-challenged (−) piglets fed* ad libitum *or restricted diets*[1]

	CTAD −	CTAD +	CTRE −	CTRE +	PEAD −	PEAD +	PERE −	PERE +	s.e.m.	Response			
Lactobacillus													
Day 0[2]	7.89	8.23	8.52	7.30	7.81	7.67	7.63	7.56	0.384				
Day 14	10.95[a]	11.55[a]	10.92[a]	9.42[b]	11.34[a]	11.05[a]	10.64[ab]	11.34[a]	0.485	R*	T × R × Ch**		
Day 35	8.46	8.57	9.02	8.90	8.75	8.67	8.97	8.96	0.208	R**			
Clostridia													
Day 0	6.08	6.64	6.29	6.43	6.05	7.07	5.69	5.41	0.504				
Day 14	2.47	2.52	2.37	2.20	2.23	2.21	1.96	2.11	0.402				
Day 35	2.30	3.28	2.20	2.42	2.40	2.19	1.96	2.10	0.398				
Enterobacteriaceae													
Day 0	6.60	7.57	7.48	6.63	7.40	7.17	5.99	6.88	0.539				
Day 14	7.88	8.90	7.81	7.92	8.12	8.01	7.55	8.27	0.541				
Day 35	4.92[a]	6.21[b]	6.38[b]	6.36[b]	4.63[a]	5.28[a]	4.97[a]	6.52[b]	0.322	T***	R***	Ch***	T × R × Ch**
E. coli													
Day 0	6.35	6.13	6.53	6.81	6.03	6.26	5.91	6.03	0.445				
Day 14	6.42	6.94	5.36	6.29	4.86	5.85	4.52	6.17	0.620	T**	Ch**		
Day 35	4.17	4.32	5.27	5.19	2.64	3.69	4.36	4.53	0.529	T**	R***		

CT = water without supplementation; PE = 8 μl/kg per day PE in drinking water; T = treatment (CT *v.* PE); AD = *ad libitum* regimen; RE = restricted regimen; R = regimen (AD *v.* RE); +/− = presence or absence of *E. coli* challenge; Ch = challenge (sham (−) *v.* infected (+)); T × R = interaction between treatment and regimen; T × Ch = interaction between treatment and challenge; R × Ch = interaction between regimen and challenge; T × R × Ch = interaction between treatment, regimen and challenge.
[a,b]Means listed in the same row with different superscripts are significantly different ($P < 0.05$).
[1]$n = 48$ (6 pens/treatment). Piglets were weaned at 24 days of age, and half of the piglets were orally challenged with *E. coli* on day 9 of the trial.
[2]day of the trial.
*$P \leqslant 0.10$, **$P \leqslant 0.05$, ***$P \leqslant 0.01$.

Table 5 *Effects of plant extract (PE) supplementation on ileum histometric parameters in* Escherichia coli*-challenged (+) and non-challenged (−) piglets fed a restricted diet*[1]

	CT −	CT +	PE −	PE +	s.e.m.	Response
Villus height (V) (μm)	361	357	350	376	13.2	
Crypt depth (C) (μm)	296[ab]	285[ab]	277[a]	304[b]	8.2	T × Ch**
V : C	1.23	1.27	1.27	1.25	0.038	
Total area of follicles (mm^2)	0.45	0.44	0.36	0.41	0.046	
Medulla area of follicles (mm^2)	0.16	0.15	0.13	0.14	0.020	
Corona area of follicles (mm^2)	0.11	0.11	0.09	0.10	0.015	
Cortex area of follicles (mm^2)	0.18	0.18	0.14	0.17	0.017	
Lymphatic follicle number (n/mm^2 mucosa)	1.43	1.53	1.45	1.46	0.104	
Macrophage number (n/mm^2 mucosa)	111	175	130	128	21.9	

CT = water without supplementation; PE = 8 μl/kg per day PE in drinking water; T = treatment (CT *v.* PE); +/− = presence or absence of *E. coli* challenge; Ch = challenge (sham (−) *v.* infected (+)); T × Ch = interaction between treatment and challenge.
[a,b]Means listed in the same row with different superscripts are significantly different ($P < 0.05$).
[1]$n = 24$ (6 piglets/treatment). Piglets were weaned at 24 days of age, and half of the piglets were orally challenged with *E. coli* on day 9 of the trial; 24 piglets with the restricted regimen (6 piglets/treatment) were slaughtered at the end of the trial.
**$P \leqslant 0.05$.

and < 0.01, respectively), whereas a slight decrease was observed in *Lactobacillus* population in piglets with RE feeding compared with animals fed AD on day 14 ($P = 0.07$). The treatment × regimen × challenge interactions for the *Lactobacillus* counts on day 14 ($P = 0.03$) indicate that PE supplementation increased the population in challenged piglets with RE diets but had no effect on other groups. The *Enterobacteriaceae* population was also affected by PE, regimen and challenge on day 35 (treatment × regimen × challenge, $P = 0.02$). The interaction was because of the differences between CTAD + and PEAD + (6.21 *v.* 5.28 log_{10} cfu/g), and between CTRE − and PERE − (6.38 *v.* 4.97 log_{10} cfu/g), but there were no differences in other groups.

Ileum histology and histometry

The effects of PE on ileum histological and histometric parameters were examined only for the RE feeding groups,

Table 6 *Effects of plant extract (PE) supplementation on intestinal mucosa inflammatory parameters in* Escherichia coli*-challenged (+) and non-challenged (−) piglets fed a restricted diet*[1]

Item	CT −	CT +	PE −	PE +	s.e.m.	Response
MPO (U/g)	1.11	2.31	1.66	2.26	0.241	Ch***
NO (μmol/g protein)	10.85	8.62	11.14	9.62	1.465	
iNOS (U/mg protein)	0.62	0.65	0.73	0.71	0.056	

MPO = myeloperoxidase; NO = nitric oxide; iNOS = inducible nitric oxide synthase; CT = water without supplementation; PE = 8 μl/kg per day PE in drinking water; T = treatment (CT *v.* PE); +/− = presence or absence, respectively, of challenge with *E. coli*; Ch = challenge (sham (−) *v.* infected (+)); T × Ch = interaction between treatment and challenge.
[1] $n = 24$ (6 piglets/treatment). Piglets were weaned at 24 days of age, and half of the piglets were orally challenged with *E. coli* on day 9 of the trial; 24 piglets with the restricted regimen (6 piglets/treatment) were slaughtered at the end of the trial.
*** $P \leqslant 0.01$.

considering both the challenged and non-challenged piglets (Supplementary Figures S1, S2; Table 5). In the ilea of *E. coli*-challenged animals without PE supplementation, signs of chronic enteritis were observed, such as irregular and flattened enterocytes at the villus tips, confluent villi at the apical zones, and hyperaemia, oedema and inflammatory cell infiltration in the lamina propria. The intestinal structure of *E. coli*-challenged pigs that had consumed the PE was normal in both the epithelium and the lamina propria. There was no PE effect ($P > 0.05$) or challenge effect ($P > 0.05$) on villus height, crypt depth, V : C ratio, total area of follicles, medulla area of follicles, corona area of follicles, cortex area of follicles, lymphatic follicle number or macrophage numbers in the intestinal mucosa. However, crypt depth in the ileum was affected by PE administration and challenge (treatment × challenge, $P = 0.03$), which was because of a difference between non-challenged piglets ($n = 277$) and challenged piglets ($n = 304$) for those receiving the PE treatment, but not for those in the CT groups.

Intestinal inflammatory parameters

The effects of PE on intestinal inflammatory parameters in *E. coli*-challenged and non-challenged piglets that were fed a RE diet are shown in Table 6. In the *E. coli*-challenged animals that were fed a RE diet, MPO activity in the ileum was increased ($P < 0.01$) compared with non-challenged piglets, whereas NO and iNOS were not affected ($P > 0.05$). PE treatment had no effect on these inflammatory parameters ($P > 0.05$).

Discussion

The objectives of the present study were to determine whether a novel PE product added to the drinking water of weaned piglets under different feeding regimens would affect growth performance and gut health and to determine whether this supplement could protect piglets against an *E. coli* challenge. In our study, PE did not affect growth performance during the overall period of the trial, but the PE piglets did show an enhanced ADG during the last week of the trial period, and they showed improvements in feed efficiency during the 2nd week and the last week. These results suggest that PE may allow piglets to better respond to stress in the post-weaning period, when impaired piglet performance is most likely to occur. Previous studies regarding the effects of PEs on growth performance in piglets have had inconsistent results. Increased feed intake, ADG and feed to gain ratio were reported by several authors (Kim *et al.*, 2004; Lien *et al.*, 2007), but other groups observed no effects on piglet growth performance (Nofrarías *et al.*, 2006; Liu *et al.*, 2013). These contradictory results may be explained by the PEs used, the compositions of the extracts, the dietary doses tested, the route of administration or the different experimental approaches used to test the effects of the substances (Windisch *et al.*, 2008).

Infections and post-weaning diarrhoea are major causes of mortality and morbidity worldwide, and they are estimated to account for as much as 50% of economic losses in the production of weaned pigs (Cutler *et al.*, 2007). In the current study, *E. coli* challenge significantly impaired piglet performance, resulting in reduced ADG and G : F ratio in the week after the challenge and, consequently, a reduced ADG in the following week. These results are in agreement with those of other authors (Liu *et al.*, 2010; Song *et al.*, 2012; Liu *et al.*, 2013). In our study, PE supplementation improved G : F only in non-challenged piglets with an AD feeding regimen; no further beneficial effect on growth performance was found during the enteric *E. coli* challenge. The *E. coli* challenge was successful in causing a mild infection. Increased faecal consistency and diarrhoea frequency were found up to 7 days after the challenge. The increases in faecal consistency scores are typical of an *E. coli* challenge model (Kiarie *et al.*, 2011; Nyachoti *et al.*, 2011). The present experiment clearly shows a reduction in the faecal consistency scores and diarrhoea frequency owing to supplementation of PE to weaned pigs subjected to the *E. coli* challenge. The effect is particularly strong within 1 week of the *E. coli* challenge. These findings are in agreement with one recent study that showed an anti-diarrhoeal effect in piglets fed PE (Liu *et al.*, 2013). The effect on faecal consistency may be related to an improved gastrointestinal microbial ecosystem in the PE-supplemented piglets. In the current study, we focused on *Lactobacillus*, *Clostridia*, total *Enterobacteriaeae* and *E. coli* because these were found to be important for intestinal health in swine (Pluske *et al.*, 2002). A positive effect was observed after PE supplementation; the *Enterobacteriaceae* and *E. coli* populations were reduced. However, PE did not affect the *Clostridia* population, which may be because of the low basal level of *Clostridia* in the intestines of piglets after weaning (Risley *et al.*, 1992). In addition, PE supplementation increased the *Lactobacillus* population in the challenged piglets that were fed a RE diet on day 14 of the trial. The mechanisms by which PEs may exert positive effects on gut populations are not yet clearly understood, although *in vitro* studies have demonstrated that the anti-microbial activities

of herbs and spices can provide protection against important pathogens (Burt, 2004; Özer *et al.*, 2007). Some reports describe the effects of green tea and pomegranate peel extracts. Tea polyphenols have been shown to exert anti-bacterial activities against human and animal disease-causing bacteria, phytopathogenic bacteria and food-borne bacteria (Sakanaka *et al.*, 2000), although the potency of the polyphenols is dependent on the bacterial species (Su *et al.*, 2008; Archana and Abraham, 2011). Recently, another hypothesis about the possible mechanism of action of polyphenols was reported by Wu and Wu (2012), who demonstrated that the same PE mix used in our study was capable of binding lipopolysaccharide, the major component of bacterial endotoxins. *In vivo* studies regarding the effects of PEs on gut microbes are less common. In humans, Goto *et al.* (1999) found that consumption of green tea selectively promoted the growth of *Bifidobacterium* and *Lactobacillus* in the gut walls of elderly residents in a long-term care facility. Hagmüller *et al.* (2006) did not find any significant differences in the shedding of haemolysing *E. coli* in weanling piglets fed *Thymi herba* (*Thymus vulgaris*, rubbed).

The decrease in ADFI and ADG as a consequence of feed restriction observed in this study has been previously reported (Lovatto *et al.*, 2006; Pastorelli *et al.*, 2012). However, the RE feeding regimen tended to improve feed efficiency. Our results showed that RE feeding led to reduced food intake during most periods of the trial, but it increased the G : F ratio compared with the AD group from day 21 to day 28. Moreover, there was a significant treatment × regimen interaction for ADG and G : F ratio during the 1st week after weaning, and lower ADG and G : F ratio values were observed in the PE treatment groups. These unexpected results, which were owing to reductions in ADG and G : F ratio in the RE piglets receiving PE compared with the piglets given water without PE supplementation, may indicate that this PE product cannot increase growth performance for piglets with a RE feeding regimen in the short post-weaning period. Similar results have been found by Daza *et al.* (2003), who reported that pigs fed a RE diet reduced their feed intake but had a feed conversion ratio similar to that of pigs fed AD. Rantzer *et al.* (1996) reported that, during the period of feeding restriction, the RE piglets had a lower diarrhoea score than those fed AD. The faecal scores of animals in this study were not influenced by feeding regimen, which indicates that feeding restriction did not impair their health status. Some previous studies observed that feed restriction resulted in a reduced health risk index (mortality + morbidity rates) in weaned rabbits compared with animals fed AD (Gidenne *et al.*, 2009), which could be explained by a decrease in the flow of nutrients to the caecum that would reduce the proliferation of pathogenic bacteria. Gidenne and Feugier (2009) found that a 20% reduction in feeding increased the caecal concentration of volatile fatty acids and acidified caecal content while enhancing bacterial fibrolytic activity. In the present study, feed restriction increased the faecal populations of *Lactobacillus*, *Enterobacteriaceae* and *E. coli* on day 35, which is different from the observations of piglets fed a RE diet by Rantzer *et al.* (1996). Lázaro *et al.* (2004) reported that feed restriction and enzyme supplementation reduced the magnitude of problems in broiler chicks, especially jejunum viscosity problems, owing to the presence of rye in feed. In addition, feed restriction implemented by decreasing the time for feeding would be aimed at precisely evaluating the utilisation of the product in the water and correlating the product effects with gut histomorphology.

Intestinal growth and development are critical for the optimal performance of piglets. After weaning, there are marked changes in the histology and biochemistry of the small intestine, including villous atrophy and crypt hyperplasia, which may cause decreased digestive and absorptive capacities and contribute to post-weaning diarrhoea (Pluske *et al.*, 1997). In our study, *E. coli* challenge induced morphological changes in the ileum, which showed signs of chronic enteritis such as irregular and flattened enterocytes at the villus tips and confluent villi at the apical zones, as well as hyperaemia, oedema and inflammatory cell infiltration in the lamina propria. The intestinal structure of *E. coli*-challenged pigs given PE supplementation was normal in both the epithelium and the lamina propria. Morphological changes in the gastrointestinal tissues occurring after phytogenic feed supplementation may provide further information about the possible benefits for gut health. The available literature does not provide a consistent picture. In our study, PE supplementation had no effect on villus height, crypt depth or V/C ratio, which is in agreement with previous observations of piglets supplemented with different PEs (Manzanilla *et al.*, 2006; Nofrarías *et al.*, 2006; Sehm *et al.*, 2007). In contrast, other authors observed an increase in villus height in the duodenum, jejunum and ileum, and a decrease in the crypt depth in response to dietary supplementation of *Acanthopanax senticosus* extract (Fang *et al.*, 2009). The interaction between treatment and challenge indicate that PE supplementation kept the V : C ratio of *E. coli*-challenged animals at a normal level by increasing villus height and crypt depth. PEs have been reported by several authors to affect the immune system of the intestine. Nofrarías *et al.* (2006) and Manzanilla *et al.* (2006) found reduced counts of intraepithelial lymphocytes in the proximal jejunum after supplementation with carvacrol, cinnamaldehyde and capsicum oleoresin. Sehm *et al.* (2007), after administration of apple pomace or red-wine pomace, observed enlargement of the ileum and the incorporation of Peyer's patches. These results may indicate an activating and/or modulating effect of PEs on the gut immune system, which may increase an animal's ability to cope with stress and reduce the effects of *E. coli* challenge. Dietary supplementation with PEs may modulate immune functions (Liu *et al.*, 2013) to reduce inflammation in the small-intestinal mucosa, which often occurs in weanling piglets. In the present study, challenge with *E. coli* caused an increase in MPO, indicating the involvement of the inflammatory response. Similarly, Steadman *et al.* (1988) showed that human polymorphonuclear leukocytes challenged with defined strains of

E. coli could release significant amounts of MPO. However, in the present study, the administration of PEs had no effect on gut inflammatory parameters.

Conclusions

Our results show that the use of a PE product based on green tea leaves (*C. sinensis*) and pomegranate rinds (*P. granatum*) improved gut health and microbial ecology in weanling piglets, thus reducing the severity of an *E. coli* challenge. This may have important implications for nutritional management on conventional farms, where specific PE supplementation could enable piglets to resist the infections that are often associated with weaning.

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Supplementary material

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