FINAL REPORT

BONIFF-SMEC: AN IN-FIELD PRACTICAL DELIVERY MECHANISM FOR IMPROVED WEANER PIGLET PERFORMANCE

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Executive Summary

This proof-of-concept experiment aimed to test a bromelain-based formulation (BONIFF) in combination with a semi-moist extruded creep (SMEC) feed in weaning pigs under an enterotoxigenic F4 *Escherichia coli* (F4-ETEC) challenge to determine the efficacy of this combined formulation on aspects of pig performance and enteric health after weaning. The experiment, using 100 newly-weaned male pigs weaned at ~21 days of age obtained from a commercial farm, was a randomised block design that comprised five treatments, being:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

The standard diet and the BONIFF/SMEC diets did not contain a pharmacological level of ZnO nor a commercial level of organic acid products (organic acids for manufacturing purposes only were added), whereas the SMEC diet alone (Treatment 5) contained commercially relevant levels of ZnO, organic acids and phytogenic compounds. Pigs were kept in groups of 5 pigs per pen with 4 pens allocated per dietary treatment (n = 20) in a building maintained at ~28° C, and on days 5 and 6 after weaning, were inoculated with F4-ETEC or were sham-challenged. Monitoring of production variables and measurements of enteric health, including post-weaning diarrhoea (PWD) and medications administered therapeutically for PWD, were recorded. Due to unforeseen circumstances preventing the selection of piglets from the original farm for this experiment, pre-weaning assessment of MUC4+ susceptibility could not be conducted and hence pigs were not allocated to treatment using this factor. Data were statistically analysed using SPSS.

The BONIFF preparation was found to be stable on the SMEC pellets from the time of delivery to the end of the experiment, a period of 6-7 weeks, in March/April 2021. Stability studies have continued beyond the trial period and continue to demonstrate good stability. This indicates that post-extrusion coating of BONIFF can viably be done.

Post-weaning diarrhoea occurred in all treatments, irrespective of F4-ETEC challenge or sham challenge, and ranged from 40% (Standard diet) to 90% [BONIFF-SMEC diet and SMEC (only) diets]. Unfortunately, the incapacity to screen pigs for MUC4+ susceptibility/resistance before the study commenced meant that an even number of pigs allocated between treatments could not occur. There was no major mortality observed in this experiment (3%), and it was not attributable to any of the treatments offered.

Pigs fed a BONIFF-SMEC diet, with or without F4-ETEC inoculation, and pigs fed a SMEC (only) diet that comprised a pharmacological level of ZnO and commercial levels of organic acids and phytogenics, generally performed better than pigs offered a Standard diet, also irrespective of with or without F4-ETEC inoculation. This period of greater performance generally coincided with the days immediately following the F4-ETEC challenge. Pigs fed the BONIFF-SMEC diet performed similarly to pigs fed the SMEC (only) diet comprising commercially relevant levels of ZnO and organic acids and phytogenic products.

Pigs fed BONIFF-SMEC (irrespective of F4-ETEC or sham-challenge) and SMEC (only) generally showed higher values for faecal consistency and the diarrhoea index throughout the study, indicative of looser faeces and more diarrhoea, compared to the pigs offered the Standard diets. More therapeutic antibiotic administrations were also required. However,

pigs fed Treatment 4, i.e., BONIFF-SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge, showed statistically similar *E. coli* (shedding) scores in the post F4-ETEC inoculation period to pigs in the two Standard diet treatments, that in turn were lower than pigs in Treatments 3 and 5.

Given the results pertaining to faecal F4-ETEC (shedding) and antibiotic medication treatments in the BONIFF-SMEC diet not challenged with ETEC, relative to the Standard diets with and without F4-ETEC challenge, further evaluation of this combination in a less F4-ETEC challenged environment is suggested.

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1. Introduction

This proof-of-concept study aimed to test a bromelain-based formulation (BONIFF) in combination with a semi-moist extruded creep (SMEC) feed in weaning pigs under an enterotoxigenic F4 *Escherichia coli* (F4-ETEC) challenge to determine the efficacy of this combined formulation on aspects of pig performance and enteric health after weaning.

Post-weaning diarrhoea (PWD) is one of the major problems in the Australian (and world) swine industries causing economic losses and decreases in the performance and survival of weaned pigs. The diarrhoea is caused by strains (types) of enterotoxigenic *E. coli* (ETEC) that adhere to receptors on the enterocytes and colonise the surface of the small intestine. The enterotoxins produced enhance the net secretion of water (i.e., into the intestines) to cause diarrhoea (Pluske *et al.*, 2018). Traditionally, antibiotics have been used for the prophylactic treatment of these pathogenic bacteria. However, rising concerns about antimicrobial resistance (AMR) caused by feeding antibiotics or other compounds such as zinc oxide (ZnO) and the ban of antibiotic-based growth promoters (AGP) and prophylactic antibiotic use in a growing number of countries has compelled the search for alternatives to in-feed antibiotics (Pluske, 2013). The issue of AMR is growing worldwide and therefore in the pork industry (Pollock *et al.*, 2020), alternatives to traditional preventative compounds such as ZnO and AGP are urgently needed to maintain pig health and welfare.

It is also known that diarrhoea in the post-weaning period can have a dietary and environmental aetiology, which may interact with ETEC proliferation to exacerbate the issue. Of note with respect to diet, the protein content/type and the dietary fibre content/type are well recognised as playing a role in diarrhoea in the post-weaning period (Heo *et al.*, 2013; Pluske, 2013; Pluske *et al.*, 2018). It is common practice to include pharmacological levels of zinc oxide and (or) copper sulphate, usually in association with an array of feed additives (organic acids, probiotics, essential oils and phytogenics, etc.), in diets after weaning to assist with the post-weaning challenges, including reducing or eliminating PWD.

Bromelain-based compounds, a proteolytic extract from pineapples (stems), have been shown previously to reduce PWD significantly in pigs by preventing attachment of the ETEC to the receptors in the small intestine (Mynott *et al.*, 1996). Detach® is a commercial product registered in Australia (Anatara Lifesciences) for the prevention of PWD in pigs, and has been shown to reduce PWD and provide similar protection to antimicrobial agents including ZnO (Holyoake and Mynott, 2017) as well as reduce AMR (Collins and Bowring, 2017). However, Detach® is a paste and hence requires labour effort to deliver the compound (i.e., orally) to pigs after weaning. A revised formulation (BONIFF) can be applied to a dry feed and fed to pigs after weaning, reducing the need for labour effort and simplifying the entire process. In combination with SMEC, semi-moist extruded creep feed, that has been shown to improve performance after weaning (Pork Cooperative Research Centre Final Report; 2009), BONIFF in combination with SMEC, as the practical vehicle to easily provide the BONIFF, has potential to (a) reduce PWD caused by ETEC and (b) improve performance in the post-weaning period.

2. Methodology

This experiment was approved by the Murdoch University Animal Ethics Committee (R3298/20).

Animals, experimental design, housing
On the day of weaning, approximately 21 days, and after on-farm selection for bodyweight (BW), 100 newly-weaned male pigs were transported to Murdoch University from a commercial farm. Day of weaning/day of arrival to Murdoch University was assigned as day zero. Pigs were allocated to their respective treatment groups on the basis of bodyweight (BW) between the groups and then allocated to pens according to a randomised block design. Pigs in all treatments received their allocated diet *ad libitum* for 11 days after weaning. Thereafter, they all received the same commercially available weaner diet *ad libitum*.

The five experimental diets in the study were as follows:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

Pigs were kept in groups of 5 pigs per pen with 4 pens allocated per dietary treatment (n = 20) in a building maintained at ~28° C. Temperature was decreased by ~2° C after 12 days maintained at ~26° C thereafter. Pigs were maintained within these treatment groups for 27 days after weaning, at which point the study finished. Individual pig weights and pen feed disappearance were recorded at regular intervals throughout the experiment to assess average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR; g of ADFI per g of ADG).

Diets
A total of four diets fed in pelleted form were used in the experiment:

1. Diet 1 (Treatments 1 and 2): standard diet without a pharmacological level of ZnO and commercially applicable inclusion levels of an organic acid (or acids);
2. Diet 2 (Treatments 3 and 4): SMEC without a pharmacological level of ZnO and commercially applicable inclusion levels of an organic acid (or acids);
3. Diet 3 (Treatment 5): SMEC with a pharmacological level of ZnO and commercially applicable inclusion levels of organic acids and phytogenic compounds;
4. Diet 4: commercially available weaner diet (Barastoc Pig Weaner), fed to all pigs from day 12-27 of the study.

Diet 3 was prepared with a pharmacological level of ZnO and commercially applicable inclusion levels of organic acids and phytogenic compounds, whereas the original intention of the study was for this diet to be without a pharmacological level of ZnO and commercially applicable inclusion levels of an organic acid (or acids). This, though, did allow for a direct comparison against the BONIFF-SMEC diet as both treatments were inoculated with ETEC.

Energy and nutrient specifications were as follows:

1. Diet 1: crude protein 21%; total fat 3.3%; crude fibre 2%; standardised ileal digestible (SID) lysine 1.2%; SID Lys:DE 0.086; acid detergent fibre 4.4%; Digestible Energy (DE) 14.0 MJ/kg; calcium 0.8%; available phosphorus ~0.5%. This was a diet containing wheat, soybean meal (solvent-extracted), whey powder, barley, fish meal, canola oil, bloodmeal, calcium carbonate, dicalcium phosphate, salt, lysine, methionine, threonine, tryptophan, Zinc oxide (to 150 ppm in the diet), choline chloride, and a vitamin and trace mineral premix.
2. Diet 2: crude protein 21%; DE 14 MJ/kg; SID Lysine 1.22%; SID Lys:DE 0.087; calcium 0.78%; available phosphorus 0.74%; organic acids for manufacturing purposes only (diet composition commercial-in-confidence);
3. Diet 3: crude protein 21%; DE 14 MJ/kg; SID Lysine 1.22%; SID Lys:DE 0.087; calcium 0.78%; available phosphorus 0.74%; 300 ppm nanoparticle ZnO; 0.7% blend of organic acids and phytogenic compounds (diet composition commercial-in-confidence);
4. Diet 4: crude protein 20.5%; DE 15 MJ/kg; SID Lysine 1.23%; SID Lys:DE 0.082; calcium 0.82%; available phosphorus 0.51%; 0.1% blend of organic acids and phytogenic compounds (diet composition commercial-in-confidence).

Enterotoxigenic Escherichia coli *challenge and monitoring*

All pigs were orally inoculated with 0.8 mL of F4 *E. coli* broth (serotype O149; F4; toxins LT, STa, STb, EAST) on days 4 and 5 after weaning (day of weaning was assigned day zero). The F4 *E. coli* was encapsulated in a gelatin capsule and administered to the pigs as per previously described (Sterndale *et al*., 2019a). Pigs that had clinical diarrhoea (FC>4) after the first capsule of ETEC were not given a second capsule. Unchallenged pigs received a sham (orally administered) challenge of physiological saline. On day 4, the first day of inoculation, the concentration of ETEC given was 1.2×10^9 CFU (colony forming units)/mL and the total concentration of ETEC given in the two capsules was 9.6×10^8 CFU. On day 5, the second day of inoculation, the concentration of ETEC given was 8.4×10^8 CFU/mL and the total concentration of ETEC given in the two capsules was 6.72×10^8 CFU.

Faecal consistency (FC), diarrhoea index [(total number of days with score 4 diarrhoea/number of days) x 100; DI] and the number of therapeutic antibiotic treatments (medications) were recorded. Faecal consistency was visually examined at the same time each morning by the same person on days 1 to 21, and was scored on a scale of 1 to 5 as follows: 1, dry and granulated; 2, dry and firm shaped; 3, moist and soft with largely retained shape; 4, pasty diarrhoea and 5, watery diarrhoea.

Post-weaning diarrhoea was assessed as being when a pig developed pasty or watery faecal consistency (FC≥4) accompanied by a stained perineum, for two

consecutive days. All pigs were swabbed upon arrival to determine *E. coli* shedding by inserting an alginated tipped swab into the anus. This was repeated on days 1, 4, 6, 7, 8 and 13 after weaning. Swabs were cultured onto sheep blood (50 ml/l) agar plates and incubated overnight at 37° C. A quantitative assessment of the proportion of *E. coli* recovered on each blood plate was made according to the number of sections (0 to 5) expressing continued streaks of clearing haemolysis around colonies displaying morphology characteristic of *E. coli*, where 0 = no growth and 5 = confluent growth to the last streak.

Pigs that developed a FC≥4 for two consecutive days or that were clinically unwell on the first day of developing diarrhoea were treated with appropriate antibiotics as recommended by a veterinarian, and the treatment continued until the diarrhoea stopped. The medications given (intramuscularly) were as follows:

1. Betamox-LA (active ingredient amoxycillin - 150 mg/ml; administered at 1 ml/10 kg BW every second day);
2. Moxylan (active ingredient amoxycillin - 150 mg/ml; administered at 1 ml/20 kg BW for 3-5 days);
3. Trisoprim (active ingredient sulphadiazine/trimethropim 400 mg/ml and 80 mg/ml; administered at 1.5 ml/30 kg BW every day until signs abated).

MUC4+ susceptibility

Due to unforeseen circumstances preventing the selection of piglets from the original farm for this experiment, pre-weaning assessment of MUC4+ susceptibility could not be conducted. Rather, MUC4+ assessment was conducted at the conclusion to the experiment according to the procedures of Sterndale *et al*. (2019b).

Statistical analyses

Continuous variables were analysed using the MIXED procedure of SPSS (version 27, Armonk NY, USA). Two separate comparisons were made to compare the different treatment structures. First, a 2 x 2 comparison was made between treatments 1-4, with diet type and ETEC challenge as fixed factors in a full factorial model and Block included in the model as a fixed (blocking) factor (y = Diet type [fixed] + ETEC challenge [fixed] + Diet type*ETEC Challenge [fixed] + Block [fixed]). Individual pig was the experimental unit for all measures except average daily feed intake (ADFI) and feed conversion ratio (FCR), which were calculated on a per pen basis, and hence pen was the experimental unit.

The second comparison carried out was a five-treatment comparison, where all treatment groups had the same initial group size (n = 20 pigs). The model used in this case was y = Treatment (fixed) + Block (fixed). For all analyses, pairwise comparisons were made between all treatment groups using the COMPARE function of the MIXED procedure, with a Bonferroni adjustment for multiple comparisons. Categorical variables were analysed using Chi-square for the five-treatment comparisons.

3. Outcomes

Three pigs did not complete the experiment. One pig (Treatment 3) died at day 8 of the study from septicaemia due to the F4-ETEC challenge, one pig (Treatment 2) died at day 15 of the study due to meningitis [*Streptococcus suis*, secondary to *Haemophilus parasuis* (Glassers Disease)], and one pig (Treatment 3) was removed and humanely euthanised because of musculoskeletal injury. All pigs underwent a post-mortem done by a veterinarian.

Application and stability of BONIFF

The B0NIFF (Batch 05-21) was provided with instructions to dissolve 45 grams in 890 ml of water. This formulation was suitable for coating 100 kg SMEC by spray coating. Ridley applied to coating to SMEC in their Queensland facilities and after a curing period of 30 days sampled the material for release. At release, the SMEC had an active coating of 0.289 +/- 0.022 micromoles/min/gram.

Samples were taken on arrival of the coated SMEC at Murdoch University, at the start of the trial (D0), at weighing day (D11), and at the end of the experiment. Analysis of the Day 11 sample showed an activity of 0.292 +/- 0.036 micromoles/min/gram. Analysis of samples from the end of the trial showed an activity of 0.257 +/- 0.036 micromoles/min/gm. From the data, we conclude that the activity of the bromelain remained a constant over the period of the trial.

There was some variability within the assay data. In part this was due to the variability in the assay procedure since only 6-10 pellets are assayed. If spraying is not completely uniform, some of the pellets may be under or over coated. This could no doubt be optimised in scaleup. However, given the volume of SMEC consumed by each piglet, the variability is more a laboratory artefact than a reflection of actual dosing.

Production performance –all experimental treatments

Between d 7-11 of the study, pigs in treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) grew faster (P = 0.016) than pigs in treatment 1 (Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge). Between d 12-13 of the study, pigs in treatment 3 (BONIFF/SMEC fed for days 1-11 after weaning, F4-ETEC challenge) grew faster (P = 0.024) than pigs in treatment 1 (Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge) (Table 1).

Between d 0-11 of the study, there was a strong trend (P = 0.054) for pigs in treatments 4 and 5 (BONIFF/SMEC fed for days 1-11 after weaning NO F4-ETEC challenge; SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge) to outperform all other treatments. For d 12-20 of the study, there was a trend (P = 0.090) for pigs in treatments 3 and 5 (BONIFF/SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge; SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge) to grow faster than all other pigs (Table 1).

There were some statistically significant effects of block, but no treatment x block interactions were observed (Table 1).

Using pen as the experimental unit of replication, there were no statistical differences in any of the production variables between the treatments (Table 2).

Table 1. Bodyweight (BW) and average daily gain (ADG) of pigs fed different diets during the experiment. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

		Treatment[A]					*P* values	
Variable		**1**	**2**	**3**	**4**	**5**	**Treatment**	**Block**
BW, kg	*n*	20	20	20	20	20		
d0		6.64	6.63	6.63	6.66	6.67	1.00	0.96
	SE	0.20	0.20	0.20	0.20	0.20		
d6		6.84	6.9	6.81	6.95	7.07	0.90	0.77
	SE	0.20	0.20	0.20	0.20	0.20		
d11		7.45	7.71	7.64	8.02	8.03	0.37	0.44
	SE	0.24	0.24	0.25	0.24	0.24		
d13		7.88	8.22	8.38	8.71	8.67	0.17	0.19
	SE	0.26	0.26	0.27	0.27	0.26		
d20		10.3	10.3	10.9	11.0	11.3	0.26	0.10
	SE	0.4	0.4	0.4	0.4	0.4		
d27		13.4	13.5	14.3	14.2	15.0	0.22	0.067
	SE	0.5	0.6	0.6	0.5	0.5		
ADG, g								
d0-6		32.5	44.8	29.9	47.5	66.7	0.42	0.21
	SE	14.7	14.7	14.7	14.8	14.7		
d7-11		122[a]	162[ab]	158[ab]	215[b]	193[ab]	0.016	0.10
	SE	20	20	20	20	20		
d12-13		220[a]	252[ab]	373[b]	344[ab]	320[ab]	0.024	0.038
	SE	37	37	38	37	37		
d14-20		344	293	355	325	377	0.21	0.17
	SE	26	26	26	26	26		
d21-27		439	460	481	464	525	0.38	0.14
	SE	31	32	33	31	31		
d0-11		73	98	89	124	124	0.054	0.11
	SE	14	14	15	14	14		
d12-20		317	286	359	329	364	0.090	0.029
	SE	22	23	23	22	22		
d12-27		370	362	416	388	435	0.17	0.050
	SE	24	24	25	24	24		
d0-27		249	254	282	280	308	0.14	0.032
	SE	18	18	19	18	18		

[A]Treatments:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

[a,b] Values in the same row not having the same superscript are significantly different.

Table 2. Bodyweight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) of pigs fed different diets during the experiment. Data are values from pens as the experimental unit of replication with standard error of the mean (SEM).

	Treatment					SEM	*P* values	
Variable	**1**	**2**	**3**	**4**	**5**		**Treatment**	**Block**
BW, kg								
d0	6.6	6.6	6.6	6.7	6.7	0.03	0.88	0.027
d6	6.8	6.9	6.8	6.9	7.1	0.10	0.44	0.38
d11	7.5	7.7	7.6	8.0	8.0	0.18	0.18	0.36
d13	7.9	8.2	8.4	8.7	8.7	0.23	0.13	0.23
d20	10.3	10.3	10.9	10.9	11.3	0.3	0.24	0.17
d27	13.4	13.5	14.4	14.1	15.0	0.6	0.29	0.16
ADG, g								
d0-6	33	45	30	46	67	18.2	0.65	0.54
d7-11	122	162	156	212	193	25	0.17	0.31
d12-13	220	252	371	337	320	46	0.19	0.24
d14-20	344	293	360	321	377	38	0.57	0.58
d21-27	439	461	483	461	525	34	0.49	0.26
d0-11	73	98	88	121	124	17	0.22	0.34
d12-20	317	285	362	325	364	30	0.36	0.30
d12-27	370	362	417	385	435	29	0.39	0.25
d0-27	249	255	282	277	308	20	0.31	0.17
ADFI, g								
d0-6	88	96	91	110	119	13	0.45	0.61
d7-11	203	223	225	262	257	21	0.28	0.28
d12-13	308	346	354	380	391	27	0.27	0.16
d14-20	429	415	478	464	503	27	0.21	0.11
d21-27	651	627	682	633	740	44	0.40	0.36
d0-11	140	154	151	179	182	16	0.32	0.37
d12-20	402	399	450	446	478	24	0.16	0.072
d12-27	511	494	551	528	593	32	0.28	0.19
d0-27	360	353	383	386	426	23	0.25	0.14
FCR, g:g								
d0-6	3.27	3.84	-1.07	4.37	2.70	2.16	0.45	0.68
d7-11	1.94	1.38	1.64	1.25	1.35	0.22	0.24	0.27
d12-13	1.53	1.51	0.96	1.14	1.51	0.28	0.50	0.71
d14-20	1.26	1.51	1.35	1.50	1.34	0.13	0.58	0.78
d21-27	1.49	1.37	1.42	1.38	1.42	0.05	0.43	0.60
d0-11	2.09	1.58	1.98	1.52	1.47	0.20	0.14	0.37
d12-20	1.29	1.45	1.25	1.39	1.32	0.09	0.54	0.76
d12-27	1.38	1.38	1.32	1.38	1.37	0.04	0.72	0.78
d0-27	1.45	1.40	1.36	1.40	1.39	0.04	0.62	0.75

[A]Treatments:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

Production performance – 2 x 2 factorial design

Table 3 shows the data from the 2x2 factorial statistical analysis using individual pigs as the unit of replication. There were statistical trends at d 13 of the study (*P* = 0.068) and at d 20 and d 27 (*P* = 0.11 and 0.12 respectively) for pigs fed the BONIFF-SMEC diet to be heavier (approximately 5-6%) than pigs fed the Standard diet. There was no significant effect of F4-ETEC on pig BW (Table 3).

For ADG, pigs fed BONIFF-SMEC grew faster between d 7-11 and 12-13 than pigs fed the Standard diet (*P* = 0.031 and < 0.001, respectively), coinciding with the immediate post-inoculation period and the change to the new diet. For d 7-11 and d 0-11, an interaction with F4-ETEC inoculation occurred (*P* = 0.018 and *P* = 0.037, respectively), with pigs fed BONIFF-SMEC without F4-ETEC infection growing faster, and noting there was no difference in ADG between the Standard diet or the BONIFF-SMEC diet when both were inoculated with F4-ETEC (*P* > 0.05) (Table 3).

During the entire duration of the study, there was a trend (*P* = 0.10) for pigs fed BONIFF-SMEC to perform better than pigs fed the Standard diet, by ~11% (Table 3).

Data in Table 4 show the data from the 2x2 factorial statistical analysis using the pen as the unit of replication. Between d 12-13, pigs fed BONIFF-SMEC grew faster (*P* = 0.022) than pigs fed the Standard diet. This corresponded to a better FCR (*P* = 0.027) in the BONIFF-SMEC-fed pigs (Table 4).

There were no statistically significant diet x ETEC interactions (Table 4).

Table 3. Bodyweight (BW) and average daily gain (ADG) of pigs fed either the Standard diet or BONIFF-SMEC diets, and not infected or infected with F4-ETEC. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

Variable		Standard Diet		BONIFF-SMEC Diet		*P* values		
		NO F4-ETEC[A]	YES F4-ETEC	NO F4-ETEC	YES F4-ETEC	Diet	F4-ETEC	Diet*ETEC
BW, kg	*n*							
d0	80	6.6	6.6	6.7	6.6	0.95	0.92	0.95
	SE	0.20	0.20	0.20	0.20			
d6	80	6.8	6.9	7.0	6.	0.95	0.85	0.60
	SE	0.20	0.20	0.20	0.20			
d11	79	7.5	7.7	8.0	7.6	0.31	0.79	0.17
	SE	0.24	0.24	0.24	0.24			
d13	79	7.9	8.2	8.7	8.4	0.068	1.00	0.22
	SE	0.27	0.27	0.27	0.27			
d20	78	10.3	10.3	11.0	10.9	0.11	0.88	0.86
	SE	0.4	0.4	0.4	0.4			
d27	77	13.4	13.5	14.2	14.3	0.12	0.81	0.94
	SE	0.5	0.5	0.5	0.5			
ADG, g								
d0-6	80	32	45	47	30	1.00	0.86	0.31
	SE	14.5	14.5	14.5	14.5			
d7-11	79	122	162	216	158	0.031	0.67	0.018
	SE	20	20	20	21			
d12-13	79	220	252	342	375	0.001	0.38	0.97
	SE	36	36	36	37			
d14-20	78	344	294	325	354	0.42	0.68	0.12
	SE	25	26	25	26			
d21-27	77	439	461	464	480	0.47	0.53	0.91
	SE	30	31	30	32			
d0-11	79	73	98	124	88	0.16	0.71	0.037
	SE	14	14	14	15			
d12-20	78	317	286	329	359	0.057	0.99	0.17
	SE	22	22	22	22			
d12-27	77	370	363	388	415	0.13	0.66	0.45
	SE	22	23	22	24			
d0-27	77	249	254	281	281	0.10	0.86	0.88

[A]ETEC: inoculation with enterotoxigenic F4 *Escherichia coli* (refer to Methodology).

Table 4. Bodyweight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) of pigs fed either the Standard diet or BONIFF-SMEC diets, and not infected or infected with F4-ETEC. Data are presented as means and standard error of the mean (SEM). Data are values from pens as the experimental unit of replication.

Parameter	Standard Diet		BONIFF-SMEC Diet		SEM	*P* values		
	NO F4-ETEC[A]	YES F4-ETEC	NO F4-ETEC	YES F4-ETEC		Diet	F4-ETEC	Diet*ETEC
BW, kg								
d0	6.6	6.6	6.7	6.6	0.03	0.74	0.51	0.68
d6	6.8	6.9	6.9	6.8	0.11	0.94	0.80	0.40
d11	7.5	7.7	8.0	7.6	0.20	0.26	0.79	0.14
d13	7.9	8.2	8.7	8.4	0.25	0.096	0.95	0.24
d20	10.3	10.3	10.9	10.9	0.4	0.14	0.97	0.95
d27	13.4	13.5	14.1	14.4	0.6	0.20	0.74	0.97
ADG, g								
d0-6	33	45	46	30	18.9	0.96	0.92	0.48
d7-11	122	162	212	156	26	0.15	0.77	0.099
d12-13	220	252	337	371	43	0.022	0.46	0.98
d14-20	344	293	321	360	41	0.61	0.88	0.30
d21-27	439	461	461	483	35	0.55	0.54	0.99
d0-11	73.3	98.3	121.3	87.5	18.5	0.34	0.82	0.15
d12-20	317	285	325	362	32	0.22	0.93	0.31
d12-27	370	362	385	417	30	0.28	0.69	0.52
d0-27	249	255	277	282	21	0.23	0.81	0.98
ADFI, g								
d0-6	88	96	110	91	14	0.57	0.70	0.39
d7-11	203	223	262	225	22	0.19	0.71	0.22
d12-13	308	346	380	354	29	0.21	0.83	0.30
d14-20	429	415	464	478	30	0.13	1.00	0.66
d21-27	651	627	633	682	47	0.71	0.79	0.46
d0-11	140	154	179	151	17	0.31	0.67	0.25
d12-20	402	399	446	450	26	0.11	0.97	0.90
d12-27	511	494	528	551	34	0.31	0.93	0.58
d0-27	360	353	386	383	25	0.28	0.84	0.93
FCR, g:g								
d0-6	3.27	3.84	4.37	-1.07	2.22	0.41	0.30	0.21
(without outlier)	3.27	3.84	4.37	2.34 ± 1.81	1.50	0.90	0.66	0.44
d7-11	1.94	1.38	1.26	1.64	0.24	0.40	0.72	0.080
d12-13	1.53	1.51	1.14	0.96	0.18	0.027	0.58	0.65
d14-20	1.26	1.51	1.50	1.35	0.14	0.79	0.70	0.17
d21-27	1.49	1.37	1.38	1.42	0.05	0.59	0.46	0.14
d0-11	2.09	1.58	1.52	1.98	0.22	0.71	0.92	0.056
d12-20	1.29	1.45	1.39	1.25	0.10	0.65	0.88	0.16
d12-27	1.38	1.38	1.38	1.32	0.04	0.42	0.45	0.45
d0-27	1.45	1.40	1.40	1.36	0.04	0.33	0.30	0.93

[A]ETEC: inoculation with enterotoxigenic F4-*Escherichia coli* (refer to Methodology).

Faecal characteristics and post-weaning diarrhoea – all experimental treatments
Post-weaning diarrhoea occurred in all treatments, irrespective of F4-ETEC challenge of the sham challenge, and ranged from 40% (Standard diet) to 90% [BONIFF-SMEC diet and SMEC (only) diets]. Using Chi-square analysis, the percentage of pigs in all treatments classified as having post-weaning diarrhoea (PWD) over the duration of the experiment was as follows ($X^2 = 27.57$, $P < 0.001$):

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge (40%);
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge (40%);
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (100%);
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge (70%);
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (90%).

Over the entire period d 0-20, FC and the DI were highest ($P < 0.001$) in treatments 3, 4 and 5, i.e., BONIFF/SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge, BONIFF/SMEC fed for days 1-11 after weaning NO F4-ETEC challenge, and SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge, relative to pigs fed the Standard diets irrespective of F4-ETEC challenge (Table 5).

Between d 4-11, which included the F4-ETEC inoculation period, similar results were seen. Between d 12-20, after F4-ETEC inoculation and when pigs were all being offered the same commercial weaner diet, pigs in treatment 2 (Standard diet fed for days 1-11 after weaning WITH F4-ETEC challenge) had firmer faeces (lower FC; $P = 0.018$) and a lower DI ($P = 0.018$) than pigs in treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) (Table 5).

The *E. coli* score (faecal swab score) broadly reflected differences in the FC and DI, although in the post-dosing (post F4-ETEC-inoculation) and overall periods, scores were lowest and the same ($P > 0.05$) in pigs in treatments 1, 2 and 4, i.e., Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge or F4-ETEC challenge, and BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge. Pigs in treatment 4 had lower ($P < 0.05$) *E. coli* scores than pigs in treatments 3 and 5 (Table 5).

There were significant ($P < 0.05$) effects of the block on the *E. coli* score, signifying some differences between treatment allocations in this measurement (Table 5). There were some effects due to block, but no significant treatment x block interactions.

Medication treatments –all experimental treatments
The number of antibiotic treatment days given during the study is shown in Table 6. Between d 4-11 of the study, pigs in treatments 1 and 2 (Standard diet fed for days 1-11 after weaning, NO ETEC challenge; Standard diet fed for days 1-11 after weaning, F4-ETEC challenge) generally recorded less ($P = 0.004$) medications than pigs in all other treatments except for pigs in treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge). There were no statistical differences between any of the SMEC diets. Between d 4-14 of the study, pigs in treatments 1 and 2 (Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge; Standard diet fed for days 1-11 after weaning, F4-ETEC challenge) recorded less ($P < 0.05$) medications than pigs in all other treatments except for

pigs in treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) (Table 6).

There was no overall statistical difference in antibiotic treatment days for pigs on treatments 4 and 5, i.e., BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge *versus* SMEC fed for days 1-11 after weaning WITH F4-ETEC challenge (Table 6), nor between these diets and pigs in treatments 2 and 3 (Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge; BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge) (Table 6).

Using Chi-square analysis, the percentage of pigs treated with antibiotics in d 0-14 of the study was as follows ($X^2 = 23.91$, $P < 0.001$):

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge (30%);
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge (30%);
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (85%);
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge (65%);
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (80%).

Using Chi-square analysis, the percentage of pigs treated with antibiotics in d 0-20 of the study was as follows ($X^2 = 24.6$, $P < 0.001$):

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge (30%);
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge (30%);
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (85%);
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge (70%);
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (80%).

The Chi-square analysis showed that pigs receiving the Standard diet, regardless of challenge, received fewer antibiotic treatments during the experiment than pigs fed the BONIFF-SMEC (regardless of challenge) or SMEC (only) diets. Less pigs in treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) required treatment for diarrhoea with antibiotics than in treatments 3 and 5.

Table 5. Faecal consistency (FC), the diarrhoea index (DI) and the rectal swab *E. coli* score of pigs fed different diets during the experiment. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

		Treatment[A]					*P* values	
Variable		**1**	**2**	**3**	**4**	**5**	**Treatment**	**Block**
[B]***FC***								
d0-3		0.15	0.15	0.35	0.15	0.30	0.35	0.33
	SE	0.12	0.12	0.12	0.12	0.12		
d4-11		1.70^{ab}	1.55^{a}	4.21^{c}	3.30^{bc}	4.20^{c}	<0.001	0.95
	SE	0.41	0.41	0.42	0.41	0.41		
d12-20		0.65^{ab}	0.11^{a}	0.64^{ab}	1.20^{b}	1.05^{ab}	0.018	0.98
	SE	0.24	0.24	0.24	0.24	0.24		
d0-20		2.50^{a}	1.70^{a}	5.20^{b}	5.00^{b}	5.60^{b}	<0.001	0.91
	SE	0.54	0.55	0.55	0.54	0.54		
[C]***DI, %***								
d0-3		3.8	3.8	8.8	11.3	7.5	0.35	0.33
	SE	3.1	3.1	3.1	3.1	3.1		
d4-11		21.3^{ab}	19.4^{a}	52.6^{c}	41.3^{bc}	52.5^{c}	<0.001	0.95
	SE	5.1	5.1	5.3	5.1	5.1		
d12-20		7.2^{ab}	1.2^{a}	7.0^{ab}	13.3^{b}	11.6^{ab}	0.018	0.98
	SE	2.6	2.7	2.7	2.6	2.6		
d0-20		12.0^{a}	8.3^{a}	25.0^{b}	23.8^{b}	26.1^{b}	<0.001	0.92
	SE	2.6	2.6	2.6	2.6	2.6		
[D]**E. coli *score***								
Total d0,1,4		0.15	0.25	0.30	0.05	0.30	0.60	0.067
	SE	0.13	0.13	0.13	0.13	0.13		
Total d6,7,8		0.40^{a}	2.60^{ab}	4.55^{b}	1.30^{a}	4.35^{b}	<0.001	0.061
	SE	0.70	0.70	0.70	0.70	0.70		
Total d13		0.35^{a}	0.00^{b}	0.05^{ab}	0.00^{b}	0.05^{ab}	0.018	0.007
	SE	0.08	0.08	0.09	0.08	0.08		
Total post-ETEC dosing		0.75^{a}	2.48^{ab}	4.28^{b}	1.30^{a}	4.40^{b}	<0.001	0.047
	SE	0.70	0.72	0.72	0.70	0.70		
Overall total		0.90^{a}	2.75^{ab}	4.50^{b}	1.35^{a}	4.70^{b}	<0.001	0.037
	SE	0.72	0.74	0.74	0.72	0.72		

[A]Treatments:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

[B]FC: refer to Methodology for description.

[C]DI: refer to Methodology for description.

[D]E. coli score: faecal swab score (refer to Methodology for description).

[a,b] Values in the same row not having the same superscript are significantly different.

Table 6. Number of days pigs were medicated for treatment of post-weaning diarrhoea. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

		Treatment[A]					*P* values	
		1	**2**	**3**	**4**	**5**	**Treatment**	**Block**
d0-3		-	-	-	-	-		
d4-11		1.00^{a}	1.45^{ab}	3.54^{c}	2.20^{abc}	3.00^{bc}	0.004	0.31
	SE	0.51	0.51	0.52	0.51	0.51		
d12-14		0.30^{ab}	0.00^{a}	0.69^{bc}	0.90^{bc}	1.00^{c}	<0.001	0.65
	SE	0.17	0.17	0.17	0.17	0.17		
d4-14		1.30^{a}	1.45^{a}	4.23^{b}	3.10^{ab}	4.00^{b}	<0.001	0.46
	SE	0.55	0.55	0.56	0.55	0.55		
d0-14		1.30^{a}	1.45^{a}	4.23^{b}	3.10^{ab}	4.00^{b}	<0.001	0.46
	SE	0.55	0.55	0.53	0.55	0.55		
d0-20		1.35^{a}	1.25^{a}	4.23^{b}	3.20^{ab}	4.00^{b}	<0.001	0.39
	SE	0.54	0.56	0.56	0.54	0.54		
d0-27		1.35^{a}	1.25^{a}	4.10^{b}	3.45^{ab}	4.00^{b}	<0.001	0.55
	SE	0.55	0.57	0.58	0.55	0.55		

[A]Treatments:

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge;
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge;
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge;
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge;
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge.

[a,b,c] Values in the same row not having the same superscript are significantly different.

Table 7. Faecal consistency (FC), the diarrhoea index (DI) and the rectal swab *E. coli* score of pigs fed either the Standard diet or BONIFF-SMEC diets, and not infected or infected with ETEC. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

Variable	*n*	Standard Diet		BONIFF-SMEC Diet		*P* values		
		NO F4-ETEC[A]	YES F4-ETEC	NO F4-ETEC	YES F4-ETEC	Diet	F4-ETEC	Diet*ETEC
[B]***FC***								
d0-3	80	0.15	0.15	0.45	0.35	0.043	0.68	0.68
	SE	0.12	0.12	0.12	0.12			
d4-11	*79*	1.70	1.55	3.30	4.21	<0.001	0.37	0.21
	SE	0.42	0.42	0.42	0.43			
d12-20	78	0.65	0.11	1.20	0.63	0.029	0.023	0.96
	SE	0.24	0.24	0.24	0.24			
d0-20	78	2.50	1.72	4.95	5.20	<0.001	0.63	0.34
	SE	0.53	0.55	0.53	0.55			
[C]***DI, %***								
d0-3	80	3.8	3.8	11.3	8.8	0.043	0.68	0.68
	SE	3.0	3.0	3.0	3.0			
d4-11	79	21.3	19.4	54.3	52.6	<0.001	0.37	0.21
	SE	5.2	5.2	5.2	5.3			
d12-20	78	7.2	1.2	13.3	6.9	0.029	0.024	0.95
	SE	2.6	2.7	2.6	2.7			
d0-20	78	12.0	8.3	23.8	24.9	<0.001	0.62	0.35
	SE	2.5	2.6	2.5	2.6			
[D]**E. coli *score***								
Total d0,1,4	80	0.15	0.25	0.05	0.30	0.85	0.18	0.56
	SE	0.13	0.13	0.13	0.13			
Total d6,7,8	80	0.40	2.60	1.30	4.55	0.033	<0.001	0.53
	SE	0.65	0.65	0.65	0.65			
Total d13	79	0.35	0.00	0.00	0.05	0.091	0.091	0.028
	SE	0.09	0.09	0.09	0.09			
Total post-dosing	78	0.75	2.54	1.30	4.26	0.082	<0.001	0.37
	SE							
Overall total	78	0.90	2.81	1.35	4.48	0.11	<0.001	0.36
	SE	0.65	0.67	0.65	0.67			

[A]ETEC: inoculation with enterotoxigenic F4 *Escherichia coli* (refer to Methodology).

[B]FC: refer to Methodology for description.

[C]DI: refer to Methodology for description.

[D]E. coli score: faecal swab score (refer to Methodology for description).

Faecal characteristics and post-weaning diarrhoea – 2 x 2 factorial design

Table 7 shows the data from the 2x2 factorial statistical analysis using individual pigs as the unit of replication. There were no significant diet x F4-ETEC interactions for any variables except for the *E. coli* score on d 13 ($P = 0.028$), which showed the highest *E. coli* score in pigs in the Standard diet without F4-ETEC.

In general, for FC and the DI, pigs fed the BONIFF-SMEC diet had more loose faeces (higher FC) and more diarrhoea (higher DI, %) (all $P < 0.05$), as well as higher

E. coli scores (0.05 < *P* < 0.1). For d 12-20, pigs fed the Standard diet had a lower FC (*P* = 0.029) and pigs inoculated with F4-ETEC had a lower FC (*P* = 0.023) but also a lower DI (*P* = 0.024) (Table 7).

Medication treatments – 2 x 2 factorial design

There were no significant diet x F4-ETEC interactions for the number of antibiotic treatment days given during the study (Table 8). There was a trend (*P* = 0.084) in d 4-11 for pigs inoculated with F4-ETEC to have more medication treatments. For all periods of the study, pigs offered the Standard diet received less (*P* < 0.001) medication treatments for diarrhoea than pigs fed the BONIFF-SMEC diet (Table 8).

Table 8. Number of days pigs were medicated for treatment of post-weaning diarrhoea. Data are presented as means ± standard error (SE). Data are values from individual pigs as the experimental unit of replication.

Variable	*n*	Standard Diet		BONIFF-SMEC Diet		*P* values		
		NO F4-ETEC[A]	YES F4-ETEC	NO F4-ETEC	YES F4-ETEC	Diet	F4-ETEC	Diet*ETEC
d0-3	*80*	-	-	-	-			
	SE							
d4-11	*80*	1.00	1.45	2.20	3.55	0.002	0.084	0.38
	SE	0.51	0.51	0.51	0.52			
d12-14	*79*	0.30	0.00	0.90	0.69	<0.001	0.10	0.78
	SE	0.15	0.15	0.15	0.16			
d4-14	*79*	1.3	1.45	3.1	4.24	<0.001	0.25	0.38
	SE	0.55	0.55	0.55	0.57			
d0-14	*79*	1.30	1.45	3.10	4.24	<0.001	0.25	0.38
	SE	0.55	0.55	0.55	0.57			
d0-20	*78*	1.35	1.26	3.20	4.24	<0.001	0.40	0.31
	SE	0.55	0.56	0.55	0.56			
d0-27	*77*	1.35	1.25	3.45	4.10	<0.001	0.63	0.52
	SE	0.56	0.57	0.56	0.59			

[A]ETEC: inoculation with enterotoxigenic F4 *Escherichia coli* (refer to Methodology).

MUC4+ susceptibility/resistance

Using Chi-square analysis, the percentage of MUC4+-susceptible was as follows (X^2 = 23.91, *P* < 0.001):

1. Standard diet fed for days 1-11 after weaning, NO F4-ETEC challenge (30%);
2. Standard diet fed for days 1-11 after weaning, WITH F4-ETEC challenge (0%);
3. BONIFF/SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (20%);
4. BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge (20%);
5. SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge (5%).

As such, and unfortunately, it was not possible to retrospectively allocate pigs within a treatment to 'susceptible' or 'resistant' to enable statistical analyses of the variables.

4. Application of Research

The overall purpose of this proof-of-concept experiment was to evaluate the impacts of a specialised bromelain-based formulation (BONIFF) in combination with a semi-moist extruded creep (SMEC) feed in weaning pigs under an enterotoxigenic F4 *Escherichia coli* (F4-ETEC) challenge, to determine the efficacy of this combined formulation on aspects of pig performance and enteric health after weaning. Two forms of statistical analyses were undertaken to more fully examine the experimental outcomes: (1) a 2x2 factorial analysis of variance with respective factors being the diet type (Standard *versus* BONIFF-SMEC) and inoculation (F4-ETEC challenge *versus* a sham challenge), and (2) a five-treatment comparison.

Pig performance

n general, although indicating only a statistical trend ($P > 0.2$), pigs fed the BONIFF-SMEC diets and the SMEC (only) diet were heavier, by ~6-11% respectively, at the end of the experiment compared to pigs fed the Standard diet. This difference appeared to be caused by a significantly faster growth rate particularly in the periods d 7-11 and d 12-13 of the experiment that corresponded to a (numerically) higher daily feed intake in those periods. These periods coincided with the immediate time after F4-ETEC inoculation and the change on day 11 to the common weaner diet, respectively. Intriguingly, d 7-11 was the period where pigs fed BONIFF-SMEC and SMEC (only) generally showed more loose faeces (higher FC) and a higher DI, and received more medications. In these circumstances, it is evidence to the product combination (in the case of BONIFF-SMEC) that performance was not only able to be maintained but was greater than the Standard diet, bearing in mind too that none of these diets contained commercially relevant levels of antimicrobial compounds.

The growth performance data also demonstrated that pigs fed the SMEC-BONIFF diet performed equally, both with and without F4-ETEC inoculation, to pigs fed the SMEC (only) diet (Treatment 5) that comprised a pharmacological level of ZnO and levels of organic acids and phytogenics seen commercially, to assist in transitioning pigs in the post-weaning period. This suggests that at least under the conditions of this experiment, BONIFF could be considered as a replacement for these additives.

Pig enteric characteristics

The F4-ETEC challenge was able to cause PWD in all treatments, irrespective of ETEC challenge of the sham challenge. This was, in part, because there was some MUC4+ susceptibility in the sham-challenged treatments, e.g., in Treatment 1, the Standard diet fed for days 1-11 after weaning with NO F4-ETEC challenge, 30% of the pigs were retrospectively found to be susceptible. As such, it could be anticipated that some diarrhoea would have occurred in pigs in this treatment, as well as in Treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge), as per in normal commercial practice without protection from antimicrobial compounds in the diet and (or) the water. Unfortunately, the inability to screen pigs for MUC4+ susceptibility/resistance before the study meant that a uniform number of pigs allocated between treatments was not possible, nor was it possible to retrospectively examine the impacts of treatments on for example PWD

in relation to MUC4+ susceptibility/resistance because some treatments, by chance, were allocated zero (Treatment 2) or just 5% (Treatment 5) MUC4+-susceptible pigs. Nevertheless, the fact that diarrhoea also occurred in Treatments 2 and 5, with zero and 5% MUC4+-susceptibility respectively, suggests that some of the faecal characteristics observed might have been of non F4-ETEC origin, e.g., due to stress-related physiological events (Pluske *et al*., 2019), and (or) be of dietary origin or attributable to other infections (see further discussion below).

For faecal consistency (FC) and the diarrhoea index (DI), in the comparison of all five treatments (Table 5), pigs fed BONIFF-SMEC (irrespective of F4-ETEC or sham-challenge) and SMEC (only) generally showed higher values throughout the study, indicative of looser faeces and more diarrhoea, compared to the pigs offered the Standard diets. However, regarding the *E. coli* score that is indicative of β-haemolytic *E. coli* shedding, pigs in Treatment 4 (BONIFF/SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) showed statistically similar values on swabbing days 6, 7 and 8 (i.e., immediately post-inoculation), and post-inoculation and overall, to pigs offered the Standard diets with and without F4-ETEC challenge, which in turn were lower than pigs in Treatment 3 and 5. These data are consistent with the Chi-square analysis indicating that there were less pigs in Treatment 4 having PWD compared to pigs in Treatment 3 and 5 (70% *versus* 90 and 90%, respectively), albeit there being pigs in Treatment 4 with PWD than in Treatments 1 and 2 (both 40%). Less pigs in Treatment 4 also required treatment for diarrhoea with antibiotics than in Treatments 3 and 5.

Data from this study also revealed that pigs fed the SMEC-BONIFF diet WITH F4-ETEC challenge (Treatment 3) did not show any greater compromised intestinal health to pigs fed the SMEC (only) diet WITH F4-ETEC challenge (Treatment 5), which comprised a pharmacological level of ZnO and levels of organic acids and phytogenics seen commercially. This suggests that at least under the conditions of this experiment, BONIFF could be considered as a replacement for these additives during an F4-ETEC challenge.

In terms of therapeutic medication treatments for PWD, in a comparison of all five treatments (Table 6), it was evident that between d 4-11 of the study, coinciding with F4-ETEC inoculation and the diet change, pigs fed the Standard diets (Treatments 1 and 2) typically had significantly less antibiotic medication treatments for PWD than pigs in all other treatments except those in Treatment 4, i.e., BONIFF/SMEC fed for days 1-11 after weaning with NO F4-ETEC challenge. This is consistent with the information presented in Table 5 regarding *E. coli* scores, above.

The difference in *E. coli* scores, a lower (albeit not always statically significant) number of antibiotic medication treatments, and less PWD (as assessed by Chi-square analysis) between pigs in Treatment 4 (BONIFF-SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge) and those in Treatments 3 and 5 (BONIFF-SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge; SMEC fed for days 1-11 after weaning, WITH F4-ETEC challenge), suggests that, at least under the conditions of the F4-ETEC challenge used in this study, there were characteristics of the SMEC diet *per se* that may have predisposed pigs to more compromised intestinal health.

In this regard, the lack of direct associations between PWD (assessed by Chi-square analysis) and the *E. coli* score in Treatment 4, and the statistical similarities in the FC and DI between Treatments 3, 4 and 5 (Table 5), supports the notion that

the aetiology of diarrhoea may not always be linked to the presence of enteric pathogens in the post-weaning period. In Denmark, where intestinal disease in nursery pigs is the most common cause of antibiotic usage, it was reported that 1/3 of non-medicated weaner pigs had diarrhoea when clinically examined even though they were assessed as healthy by stock personnel (Weber *et al.*, 2015). These authors found there was no association between detection of pathogens and diarrhoea status of the individual pigs and between detection of pathogens in a pen and diarrhoea floor pools. In 51% of the samples from diarrhoeic pigs, pathogens were not detected. Weber *et al.* (2015) concluded that the diarrhoeic status of the pigs and diarrhoeic pools in a pen were poor indicators of intestinal infections with F4 ETEC (along with other pathogens – F18 ETEC, *L. intracellularis* and *B. pilosicoli),* and subclinical infections were common. Weber *et al.* (2015) recommended that clinical examination and counting of diarrhoea pools should be supported by microbiological testing as decision tools for initiation of batch treatments of intestinal infections in weaner pigs.

It is known that diet, for example protein content/type and dietary fibre content/type, is implicated in the aetiology of PWD in pigs and can exacerbate diarrohea under certain circumstances (Heo *et al.*, 2013; Pluske *et al.*, 2018). Diets in this experiment were formulated to be approximately similar with respect to protein content and SID lysine; however, it is unknown whether fibre types and contents, or indeed other dietary components, varied between the Standard diet and the BONIFF-SMEC/SMEC (only) diets.

In the 2x2 factorial statistical analyses (i.e., diet type *versus* with/without F4-ETEC; Tables 7 and 8), the overall lack of any significant diet type x F4-ETEC inoculation interactions signified that the effects of diet type and F4-ETEC were independent. In general, pigs inoculated with F4-ETEC and pigs fed BONIFF-SMEC showed faecal characteristics and diarrhoea reflective of more compromised enteric health, commensurate with a greater number of antibiotic medication treatments.

5. Conclusion

Post-weaning diarrhoea occurred in all treatments, irrespective of F4-ETEC challenge or sham challenge, and ranged from 40% (Standard diet) to 90% (BONIFF-SMEC diet and SMEC (only) diets). Unfortunately, the incapacity to screen pigs for MUC4+ susceptibility/resistance before the study commenced meant that an even number of pigs allocated between treatments could not occur. There was no major mortality observed in this experiment (3%), and it was not attributable to any of the treatments offered.

Pigs fed a BONIFF-SMEC diet, with or without F4-ETEC inoculation, and pigs fed a SMEC (only) diet that comprised a pharmacological level of ZnO and levels of organic acids and phytogenics seen commercially, generally performed better than pigs offered a Standard diet, also irrespective of with or without F4-ETEC inoculation. This period of greater performance generally coincided with the days immediately following the F4-ETEC challenge. Pigs fed the BONIFF-SMEC diet performed similarly to pigs fed the SMEC (only) diet comprising commercially relevant levels of ZnO and organic acids and phytogenic products.

Pigs fed BONIFF-SMEC (irrespective of F4-ETEC or sham-challenge) and SMEC (only) generally showed higher values for FC and the DI throughout the study,

indicative of looser faeces and more diarrhoea, compared to the pigs offered the Standard diets. However, pigs fed Treatment 4, i.e., BONIFF-SMEC fed for days 1-11 after weaning, NO F4-ETEC challenge, showed statistically similar *E. coli* (shedding) scores in the post F4-ETEC inoculation period to pigs in the two Standard diet treatments, that in turn were lower than pigs in Treatments 3 and 5.

Given the results pertaining to faecal *E. coli* (shedding) and antibiotic medication treatments in the BONIFF-SMEC diet not challenged with F4-ETEC, relative to the Standard diets with and without F4-ETEC challenge, further evaluation of this combination in a reduced F4-ETEC challenged environment, e.g., transient PWD, is suggested.

6. Limitations/Risks

To the application of the research findings:

1. The BONIFF coating to the SMEC pellets might not have been consistent overall. As mentioned previously, if spraying was not completely uniform, some of the pellets may be under or over coated with BONIFF. However, this could be optimised during scale-up with larger quantities.
2. Pigs in this proof-of-concept study were housed in 'ideal' conditions, e.g., constant and optimum ambient temperature, fully clean and disinfected pens, low stocking density, regular care and attention, hence the results obtained are likely not immediately transferable to commercial conditions.
3. This was a proof-of-concept study, and the prevalence and incidence of PWD under Australian conditions (and indeed, in other parts of the world) would differ. The F4-ETEC challenge in this experiment, whilst moderate (i.e., a greater number of CFU/ml would likely have caused greater mortality), may not necessarily be reflective of all commercial conditions. More mild cases of PWD, that nonetheless may still be the subject of diet/water antimicrobial schedules, could have resulted in different outcomes with the feeding of the BONIFF-SMEC diet.
4. The Standard diet and BONIFF-SMEC diets were designed to be equivalent in energy and macronutrient contents. However, it cannot be discounted that there were differences in other dietary components such as dietary fibre content/type, different amounts of (ileal) indigestible protein, that contributed to some of the differences seen in the enteric health outcomes.
5. Treatment 5 (Diet 3), the SMEC (only) diet, was originally intended to be the same composition as Treatments 3 and 4, i.e., without a pharmacological level of ZnO and commercially applicable inclusion levels of an organic acid (or acids). However, this diet was manufactured with a pharmacological level of ZnO and commercially applicable inclusion levels of organic acids and phytogenic compounds. This, therefore, could not tease out any effects SMEC alone might have on F4-ETEC challenge over the BONIFF-SMEC. However, the fact that pigs fed the SMEC-BONIFF diet (even with F4-ETEC challenge) performed equally to pigs fed the SMEC (only) diet (Treatment 5) suggests that, at least under the conditions of this experiment, BONIFF could be considered as a replacement for these additives. Nevertheless, more (commercial) evaluation would be required to test such a proposition.

6. Resource and housing/logistical constraints mean that a sixth treatment, i.e., SMEC (alone) and NO F4-ETEC challenge, could not be accommodated. Such an additional treatment would have resulted in a more balanced experimental design and a greater chance of detecting diet by F4-ETEC challenge interactions.

7. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

1. Under the conditions of this proof-of-concept study and given the experimental outcomes, BONIFF could be considered as a replacement for a non-physiological level of ZnO and higher levels of organic acids (and phytogenics) in a SMEC diet.
2. Given the results pertaining to faecal *E. coli* (shedding) and antibiotic medication treatments in the BONIFF-SMEC diet not challenged with F4-ETEC, relative to the Standard diets with and without F4-ETEC challenge, further evaluation of this combination in a less F4-ETEC challenged environment is suggested.
3. The BONIFF preparation was found to be stable on the SMEC pellets from the time of delivery to the end of the experiment, a period of 6-7 weeks, in March/April 2021. This indicates that post-extrusion coating of BONIFF can viably be done.
4. The outcomes from a current APRIL-funded project being conducted by the SunPork Group (6A-103, *Easing the transition: large piglets from large pellets*) should be monitored as this experiment, conducted under commercial conditions, seeks to evaluate a form of SMEC in more detail, and even though the formulation of SMEC and its application to piglets does vary, the principals of using a SMEC are similar. In study 6A-103, performance will be monitored from day 4 of lactation through to 7 days after weaning, before pigs are transitioned to a standardised creep feed for the following 3 weeks. Growth performance, feed disappearance and piglet treatment and mortality will be monitored across these periods, with feeding behaviour monitored to study differences in intake patterns across treatments and time.

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Appendices