2C-119: Alternatives to vitamin E for the cost-effective management of cellular antioxidant capacity in weaner pigs experimentally infected with enterotoxigenic strain of E. coli

Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

Vitamin E is often added to commercial diets for weaner pigs at levels of 50–100 IU/kg feed for its antioxidant effects. At weaning, due to a number of abrupt dietary, environmental and social changes, marked anti-inflammatory and anti-stress responses are often observed resulting in increased oxidation stress and cell damage. The vitamin E reserve in the body of piglets is known to rapidly decrease close to the deficiency level (1 mg/L in plasma) due to these stressors in the three weeks of weaning. Moreover, the reduction in the vitamin E reserve can further decrease when piglets are exposed to enteric pathogens such as *Escherichia coli* (*E. coli*). Therefore, increasing antioxidant levels in the diet for weaner pigs is generally accepted as a complementary strategy to reduce morbidity and mortality in the immediate post-weaning period. Increasing vitamin E levels in the diet to ≥ 200IU/kg of feed has reported favourable outcomes with respect to feed conversion most likely through an improvement in the anti-inflammatory response. However, recent publications indicate that a comparable level of cellular antioxidant and anti-inflammatory capacities can be achieved through the use of other catalysts such as quercetin (a plant flavonoid) or a combination of copper and vitamin C.

The aim of this project was to explore the effectiveness of alternative feed additives that can partially replace vitamin E in diets for weaner pigs while maintaining cellular antioxidant and anti-inflammatory capacities. A standard commercial inclusion level of vitamin E (70IU/kg) in the diet was compared with high inclusion levels (200IU/kg) and partial inclusion levels (50IU/kg). The partial inclusion level diets included either quercetin (30mg/kg) or a combination of copper sulphate (175ppm) and vitamin C (500mg/kg). An in-feed antibiotic was also included as another treatment given the pork industry has traditionally used in-feed antibiotics to ameliorate pathogen infection and mitigate the post-weaning malaise. Five out of the six treatment groups were challenged with a strain of enterotoxigenic *E. coli* (ETEC) one week after weaning.

There was no difference in the incidence of post-weaning diarrhoea throughout the experiment, however, pigs not challenged with ETEC and pigs challenged with ETEC *and* given an in-feed antibiotic grew the least amount of beta-haemolytic *E. coli* on sheep blood agar plates. As expected, plasma vitamin E was highest in pigs supplemented with the highest level of vitamin E (200IU/kg) and all indicators of inflammation (haptoglobin, C-reactive protein), oxidative stress (malondialdehyde, glutathione, total antioxidant capacity) as well as metabolic stress (urea) increased immediately after the *ETEC* challenge with the exception of PigMAP, where there was no change over time across treatments.

Despite the ETEC challenge inducing an inflammatory and oxidative stress response, no significant differences between the different dietary treatments for body weight, average daily gain, average daily feed intake and feed conversion ratio were appreciated. The pigs given an in-feed antibiotic did however have the lowest plasma concentration of haptoglobin and C-reactive protein compared with the other treatment groups with the exception of the pigs that were not challenged with ETEC.

Overall higher levels of vitamin E supplementation (200IU/kg) and partial replacement of vitamin E with quercetin or a combination of copper sulphate and vitamin C had no effect on the immune response or performance in pigs challenged with ETEC one week after weaning. This is in contrast to what as previously been reported and the disparity between the results could be explained by differences in experimental conditions, the selected inclusion levels for the diet supplements or the level of pathological state reached by the pigs subjected to the ETEC challenge. Nevertheless, these results do highlight the importance of the anti-

inflammatory effect of antibiotics, and understanding this effect further may assist swine industries worldwide as they explore alternatives to antibiotics.

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Introduction

In commercial piggeries, piglets are commonly exposed to weaning stress and various enteric pathogens within the facility which often cause post-weaning diarrhoea (PWD), that is sometimes accompanied with morbidity and mortality. The physiological response of pigs to stressors and infection pressure are diverse such as anti-stress and anti-inflammatory, however, one of the common results of the various physiological responses is increased oxidation stress and cell damage by the reactive oxygen species produced during the physiological defence mechanism (Kim et al., 2013). Therefore, increasing antioxidant levels in the diet for weaner pigs is generally accepted as a complementary strategy to reduce morbidity and mortality at around weaning.

Vitamin E is an antioxidant; however, the vitamin E reserve in the body of piglets is known to rapidly decrease after weaning close to the deficiency level (1 mg/L in plasma, Sivertsen et al., 2007). Moreover, the reduction in the vitamin E reserve after weaning is known to further decrease when piglets are exposed to enteric pathogens such as *Escherichia coli* (*E. coli*) (Lauridsen et al., 2011). Currently commercial diets are supplemented with 50-100 IU of vitamin E per kg diet. A previous CRC project (Project 2C-110, "Dietary manipulation of the pro-inflammatory cascade to minimise impacts on health and production indices in weaner pigs experimentally infected with an enterotoxigenic strain of *E. coli*") found the production of haptoglobin (Hp, an acute-phase protein) was reduced in *E. coli*-infected weaner pigs supplemented with 200 IU of synthetic vitamin E in the diet compared with pigs fed a 50 IU vitamin E diet (Fig. 1). A follow up study conducted on a commercial farm (CHM, Queensland) showed a significant improvement in feed utilisation efficiency in weaner pigs fed with 250 IU vitamin E compared with 75 IU vitamin E diet (Kim et al., 2016).

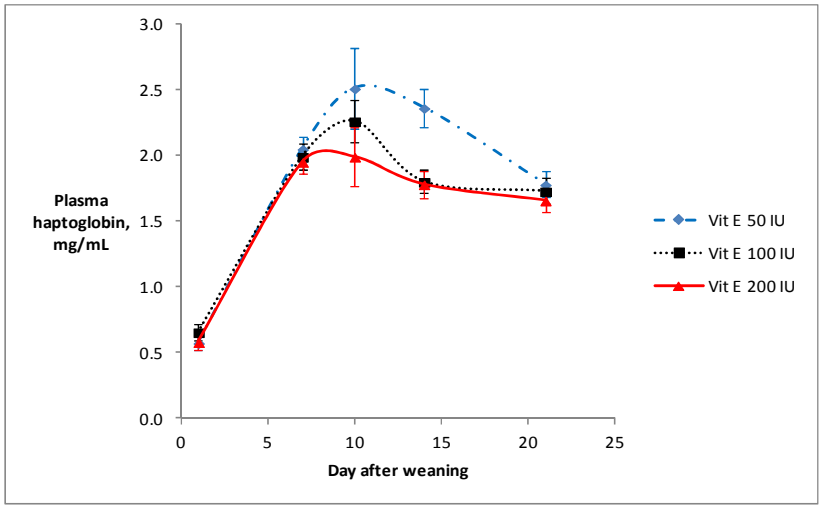

Figure 1. The effect of dietary vitamin E level on plasma haptoglobin contents after weaning. All pigs were experimentally infected with an enterotoxigenic strain of *E. coli* on day 7 (after blood sampling), 8 and 9.

However, recent publications indicate that a comparable level of cellular antioxidant and anti-inflammatory capacities to additional vitamin E supplementation can be achieved through the use of other catalysts such as quercetin (Farombi and Onyema, 2006) or a combination of copper and vitamin C (Lauridsen et al., 2000; Lauridsen and Jensen, 2005).

Quercetin is a plant flavonoid (extracted from onion, tea and berries). In an immune system-stimulated rat study it was demonstrated that supplementation of 10 mg quercetin (20 times less than vitamin E) showed comparable antioxidant capacity to either 200 mg vitamin E or C, when indices for cellular antioxidant capacity such as glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase were measured in the liver, kidney and brain (Farombi and Onyema, 2006). Increased antioxidant and anti-inflammatory capacity in rats fed quercetin also showed greater prevention from genotoxicity than a 200 mg vitamin E-treated group under experimental oxidative stress (Noroozi et al., 1998; Farombi and Onyema, 2006). The DNA damage in this rat study was measured as the appearance of micro nucleated polychromatic erythrocytes in the bone marrow, which is a standard measure of DNA damage for toxicology studies. In an *in vitro* study, quercetin also significantly decreased production of pro-inflammatory cytokines such as TNF-α and IL-6 (Chirumbolo, 2010). A recent *ex vivo* study using rat liver epithelial cells also clearly demonstrated that quercetin significantly decreased expressions of immunosuppressive molecule prostaglandin E_2 (PGE_2) and the responsible enzyme cyclooxygenase-2 (COX-2) under arsenite-induced toxicity (Lee et al., 2010).

Lauridsen and Jensen (2012) highlighted that when additional vitamin E is supplemented in the diet, vitamin E deposition principally occurs in the mitochondrial and microsomal membranes, where large amounts of reactive oxygen species are produced under stress and inflammation. An early pig study reported that co-supplementation with 200 IU vitamin E and 175 ppm copper (as Cu (II)-sulphate pentahydrate) synergistically increased vitamin E deposition in the liver (control 2.5, vit E 17.8, Cu 5.9, vit E+Cu 27.3 g/g; Lauridsen et al., 2000). It was also reported in a later study that co-supplementation of vitamin E and 500 mg vitamin C additively increased vitamin E deposition in the liver (Lauridsen and Jensen, 2005). These studies suggest that at least some proportion of dietary vitamin E can be replaced with supplementation of copper and vitamin C.

Therefore, this research aimed to evaluate quercetin and copper and vitamin C as a partial replacement of vitamin E while maintaining cellular anti-oxidant and anti-inflammatory capacities. The pork industry has traditionally used in-feed antibiotics to ameliorate pathogen infection which may have changed the whole dynamic of the digestion process by reducing both pathogenic and commensal microbiota in the gastrointestinal tract. Therefore, the treatment effects were tested against a non-infection control, an infection control, and an infection with in-feed antibiotic control to interpret data more comprehensively, and also to evaluate commercial implications.

Methodology

Aims:

To explore the effectiveness of alternative feed additives that can partially replace vitamin E in diets for weaner pigs while maintaining cellular antioxidant and anti-inflammatory capacities.

Hypotheses:

1. Supplementation of 200 IU vitamin E in *E. coli*-infected pigs would improve antioxidant and anti-inflammatory capacity compared with pigs in the infection control group, in addition to improvements in post-weaning performance.
2. Partial replacement of vitamin E with quercetin (replacing 150 IU vitamin E with 30 mg quercetin) would have comparable antioxidant and anti-inflammatory capacities to the infected pigs supplemented with 200 IU vitamin E.

3. Partial replacement of vitamin E (150 IU) with 175-ppm copper and 500 mg vitamin C would have comparable antioxidant and anti-inflammatory capacities to the infected pigs supplemented with 200 IU vitamin E.

Materials and Methods:

The experimental protocol used in this study was approved by the Department of Agriculture and Food Western Australia Animal Ethics committee (AEC 15-3-11). Animals were handled according to the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013). The experiment was conducted in November/December 2016 at the Medina Research Station in Western Australia.

Animals, experimental design, diets and housing

96 male pigs (Large White x Landrace) weighing 6.76 ± 0.82 kg were weaned from a commercial farm at 21 days of age. On the day of weaning, pigs were weighed and then transported 3 hours to Medina Research Station. The pigs were housed in individual pens in a temperature controlled room. The 16 pigs that were not infected were housed in the front section of the room and the remaining pigs were allocated to their experimental diets in a randomized block design. There were 5 different diets and a total of 6 six treatments (*n*=16).

The treatments were:

- No-infection + Control diet (basal diet contained 70 IU vitamin E) **(NI-C)**;
- Infection + Control diet **(I-C)**;
- Infection + Control + feed-grade amoxicillin (200 g/200 kg of feed) **(I-AB)**;
- Infection + Control + 200 IU vitamin E **(I- VitE)**;
- Infection + Control + 50 IU vitamin E + 30 mg quercetin **(I-Qu)**;
- Infection + Control + 50 IU vitamin E + 175 ppm copper + 500 mg vitamin C/kg diet **(I-VitC-$CuSO_4$)**.

Table 1 – Composition of control diet

Ingredients	g/kg
Barley	100.00
Wheat	351.96
Rolled oats	100.00
Soybean meal	58.46
Full fat soya	50.00
Blood meal	20.00
Fishmeal	50.00
Skim milk powder	115.82
Whey powder	100.00
Canola oil	33.21
Lysine	3.84
Methionine	2.71
Threonine	1.87
Tryptophan	0.12
Isoleucine	0.45
BJ-Pig grower plus	1.00
Limestone	6.61
Dical Phos	1.91
Salt	2.00
Choline Chloride	0.02
Calculated composition	
DE, MJ/kg	15.50
NE, NJ/kg	10.49
Protein, %	20.50
Fat, %	6.50
NDF, %	8.27
ADF, %	2.27
Available lysine, %	1.395

[1] Treatment diets were same as control with the addition of: Antibiotic = 1g/kg Amoxicillin, Vitamin E = 0.4g/kg Vitamin E (50%), Quercetin = 0.1g/kg Vitamin E (50%) + 0.03g/kg quercetin, Vitamin C + $CuSO_4$ = 0.7g/kg $CuSO_4$ (25% copper) + 0.5g/kg Vitamin C (99%) + 0.1g/kg Vitamin E (50%).

[2] BJ Grower 1 (BioJohn Pty Ltd, Belmont, WA, Australia), provided the following nutrients (per kg of air-dry diet): vitamins: A 7000 IU, D3 1400 IU, E 20 mg, K 1 mg, thiamine 1 mg, riboflavin 3 mg, pyridoxine 1.5 mg, cyanocobalamin 15 μg, calcium pantothenate 10.7 mg, folic acid 0.2 mg, niacin 12 mg, biotin 30 μg. Minerals: Co 0.2 mg (as cobalt sulfate), Cu 10 mg (as copper sulfate), I 0.5 mg (as potassium iodine), Fe 60 mg (as ferrous sulfate), Mn 40 mg (as manganous oxide), Se 0.3 mg (as sodium selenite), Zn 100 mg (as ZnO).

Pigs were individually housed and fed the experimental diets *ad libitum* from the day of weaning (day 0) until 21 days after weaning (day 21). The pens were made of metal wire-mesh with a space allowance of 0.42m^2 per pig. Each pen had a nipple water drinker and a metal feeding trough. Pigs were monitored twice daily and weighed

weekly to calculate body weight gain. Feed disappearance from each pen was recorded weekly and feed wastage was visually assessed and recorded daily to calculate feed intake and feed conversion ratio.

Oral *E. coli* dosing, faecal scoring and beta-haemolytic *E. coli* scoring

On days 7 and 8 after weaning, 80/96 pigs were infected with an enterotoxigenic strain of E. coli (ETEC, serogroup O149:K91:F4) by oral drenching of 1 ml concentrated ETEC solution with buffered saline ($2x10^{10}$ CFU/ml). Pigs in the non-infection group were given an oral dose of 1 ml sterile phosphate-buffered saline to induce the same level of handling and dosing stress subjected to the infection group. The *E. coli* solution was freshly prepared on the dosing day according to the method described by Heo et al. (2009), and oral dosing was achieved by placing the syringe at the rear of the oral cavity.

Pigs were monitored daily for the presence of diarrhoea. Faeces were scored depending on their consistency using the following criteria: 1 = firm and well-formed, 2 = soft formed faeces, 3 = faeces falling out of shape upon contact with surfaces and sloppy, 4 = watery consistency. Score 4 was considered as diarrhoea. In accordance with the animal ethics application, pigs with a faecal score 4 were treated immediately with Moxylan IM (amoxicillin 150 mg/mL, Jurox Pty Ltd., Rutherford NSW, Australia), and this was repeated daily for 3-5 days. The numbers of therapeutic antibiotic treatment were recorded throughout the experiment (equivalent to the number of pigs that developed PWD). The diarrhoea index (DI) was calculated as the mean proportion of days with diarrhoea with respect to 14 days after weaning.

Faecal beta-haemolytic *E. coli* shedding was measured on days 0, 7, 9, 10, and 14 after weaning by swabbing the rectum with a cotton bud. Faeces from the swab were incubated on sheep blood (50 ml/L) agar plates (ThermoFisher Scientific, Adelaide, South Australia). Plates were incubated overnight at 37 °C and colonies were assessed based on morphology and haemolysis. Scores were given on a scale from 0 to 5 according to the number of streaked sections containing viable haemolytic E. coli, where 0 was no growth and 5 was growth out in the fifth section of the plate (Heo et al., 2009).

Blood samples

Blood samples were collected on days 6 (pre-infection), 9 (post-infection, acute response) and 14 (post-infection, chronic response) of the experiment. Samples were collected via jugular venepuncture into lithium heparin coated tube, which were immediately placed on ice. Samples were centrifuged at 2800 x *g* for 15 minutes at room temperature. Plasma was collected and stored at either -20 degrees C or -80 degrees C.

Plasma was subsequently analysed at the Animal Health Laboratories (Department of Primary Industries and Regional Development, Western Australia) for the determination of: vitamin E, Haptoglobin (Hp), urea, MDA, and total GSH. Haptoglobin content in the plasma sample was determined using an in-house method adapted from Eckersall et al. (1991). Urea analysis was performed using the Beckman Coulter/Olympus Reagent kit (OSR6134). Vitamin E was analysed using a high-performance liquid chromatography based on techniques used by McMurray and Benchflower (1978). Malondialdehyde analysis was performed using flurometric analysis of 2-thiobabituric acid and total GSH was assayed using Abnova Glutathione Assay kit (Cat KA0800, SapphireBioscience) and analysed using the BMG labtech

POLARstar Omega (inter and intra assay <12.5%). Analysis for plasma C-reactive protein (CRP), total antioxidant capacity (TAC) and PigMAP was performed at Murdoch University using commercial available ELISA kits (CRP: DY2648, R and D systems; TAC: STA-360, Cell Biolabs, Inc.; PigMAP: PME02, AcuVet Biotech). Analysis using commercial kits was in accordance with the manufacturers' instructions.

Statistical Analyses

A one-way analysis of variance (ANOVA) was used for statistical evaluation of the performance and faecal data using SPSS (version 24, IBM corporation, Armonk, NY, USA). The pig was the experimental unit. A mixed model analysis with day as the repeated measure and treatment, day and treatment x day as fixed effects was used to analyse oxidative capacity and inflammatory markers in the blood. The pig was used as the experimental unit for the blood measures. When either the ANOVA or mixed model analysis detected a significant difference for the fixed effects, pairwise comparisons were used to examine differences between the 6 treatment groups. Residues were checked for normality and frequency data were square root transformed if needed. The distribution for the *E. coli* plate scores data were not normal and transformation of the data did not correct this. The *E. coli.* plate scores were therefore compared between treatments using a Kruskal-Wallis test with post-hoc analysis to determine which groups were different from one another. A chi-squared analysis was used to examine differences between treatments for the expression of diarrhoea over the 14 days after weaning.

Outcomes

Two animals were removed from the trial prior to the ETEC challenge due to ill-thrift and diarrhoea. Two other animals were removed from the trial after the first dose of the ETEC challenge (day 8) because of severe weight loss (>20%). One animal died on day 18 of the experiment after it failed to respond to treatment for diarrhoea. After day 14 of the experiment, the diarrhoea persisted in a large proportion of the pigs and new cases were starting. At that time, a decision was made to top dress the feed with amoxicillin (excluding the I-AB treatment group). This made no difference to the incidence of diarrhoea, therefore on day 18 of the experiment, in-water medication (amoxicillin, 20 mg/kg) was given to all pigs and the I-AB treatment group was changed to the control diet. Consequently, the performance and faecal data are only presented up to day 14 of the experiment.

There was no difference between treatments for incidence of PWD and the DI throughout the experiment ($p > 0.05$; Table 1). As expected, there was an increase in the haemolytic *E. coli* score after infection (from 0.22 to 3.83, $p < 0.001$), and while there was no difference between treatments before infection, pigs not challenged with ETEC (NI-C) and pigs given an in-feed antibiotic (I-AB) had the lowest *E. coli* scores after infection ($p < 0.001$; Table 1). The pairwise comparisons highlighted that pigs given VitC-$CuSO_4$ supplementation (I-VitC-$CuSO_4$) tended to have a lower *E. coli* plate score than the in-feed antibiotics (I-AB) (Table 1).

No significant differences between treatments for body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were appreciated on the selected measurement days (Table 2).

Table 1. Mean values for indices of post-weaning diarrhoea measured for 14 days after weaning for pigs from six different dietary treatment groups.

	Treatment[1]							
Item	**NI-C**	**I-C**	**I-AB**	**I-VitE**	**I-Qu**	**I-VitC-$CuSO_4$**	**SEM**	**P value**
% pigs with PWD[2]								
	50	60	44	29	67	56		0.38
DI[3], %								
	4.01	6.67	8.48	4.08	7.14	4.46	2.12	0.49
***E. coli* plate score[4]**								
Pre-infection	0.13	0.20	0.19	0.00	0.47	0.31	0.22	0.78
Post-infection	1.19^{a}	4.67^{b}	1.88^{ax}	5.64^{b}	6.00^{b}	4.06^{by}	1.00	<0.001

[1] NI-C = non-infection + control; I-C = infection + control; I-AB = infection + in-feed antibiotic; I- VitE = infection + 200 IU vitamin E/kg feed; I- Qu = Infection + 30 mg quercetin/kg + 50 IU vitamin E; I-VitC-CuSO4 = Infection + 50 IU vitamin C + 175 ppm copper + 500 mg vitamin C/kg feed.

[2] PWD = post-weaning diarrhea from day 0 to 14. Post-weaning diarrhea was defined as pigs having a faecal consistency score of 4. Data were tested using a chi square test.

[3] DI = diarrhoea index, mean proportion of days with diarrhoea with respect to 14 d after weaning. Data were square root transformed for statistical analysis. Raw means are expressed in the table.

[4] Mean cumulative *E. coli.* score per treatment before infection (day 0 to 7) and after infection (d 8 to 14). Data were tested non-parametrically.

[a-c] Means in a row not having the same superscript are significantly different ($p < 0.05$).

[x-y] Means in a row not having the same superscript are a trend ($p < 0.1$).

Table 2. Mean values for pig BW, ADG and ADFI for pigs from six different dietary treatment groups up to 14 days after weaning.

	Treatment[1]							
Item	NI-C	I-C	I-AB	I-VitE	I-Qu	I-VitC-$CuSO_4$	SEM	P value
BW, kg								
Day 0	6.8	6.8	6.7	6.7	6.8	6.7	0.23	1.00
Day 7	7.6	7.4	7.4	7.3	7.4	7.6	0.27	0.94
Day 14	9.9	9.4	9.3	9.3	9.1	9.7	0.38	0.65
ADG, g								
Day 0 -7	100	79	97	87	82	129	16.4	0.25
Day 7-14	323	287	269	282	253	292	29.7	0.63
Day 0-14	211	183	183	184	167	210	18.91	0.46
ADFI, g								
Day 0 -7	126	123	123	117	119	142	12.1	0.71
Day 7-14	381	326	320	336	321	365	25.4	0.35
Day 0-14	253	225	221	227	220	254	17.11	0.45
FCR, g/g								
Day 0-7	1.7	1.2	1.7	1.8	2.1	1.3	0.46	0.76
Day 7-14	1.2	1.2	1.5	1.2	1.4	1.2	0.14	0.49
Day 0-14	1.3	1.3	1.3	1.3	1.4	1.3	0.09	0.75

NI-C = non-infection + control; I-C = infection + control; I-AB = infection + in-feed antibiotic; I- VitE = infection + 200 IU vitamin E/kg feed; I- Qu = infection + 30 mg quercetin/kg + 50 IU vitamin E; I-VitC-$CuSO_4$ = infection + 50 IU vitamin C + 175 ppm copper + 500 mg vitamin C/kg.

As expected, plasma vitamin E was highest in the I-VitE treatment group (treatment effect, $p < 0.001$) (Table 3) and plasma vitamin E concentrations were higher across all treatment groups on days 9 (2 days after infection) and 14 (7 days after infection) compared with day 6 (before infection) (day effect, $p < 0.01$) (Table 3). All indicators of inflammation (Hp, CPR), oxidative stress (MDA, GSH, TAC) as well as metabolic stress (urea) increased between days 6 to 9 ($p < 0.01$, day effect) with the exception of PigMAP, where there was no change over time across treatments ($p > 0.05$) (Table 3).

While markers of inflammation (Hp, CRP) reduced back to pre-infection levels by day 14 of the experiment, markers of oxidative stress (MDA, GSH, TAC) remained increased ($p > 0.001$) on day 14 compared with pre-infection levels (day 6). These results confirm that infection with an enterotoxigenic strain of *E. coli* (ETEC) on day 7 of the experiment induced an inflammatory and oxidative stress response. While no day x treatment effects were identified for the blood measures, the I-AB pigs had the lowest plasma concentration of Hp and C-RP overall compared with the other treatment groups, with the exception of NI-C pigs having a similar Hp and CRP concentration to I-AB pigs (treatment effect, $p < 0.01$) (Table 3). There was also an overall treatment effect for MDA with I-AB pigs demonstrating the highest level of oxidative stress and the NI-C and I-C pigs the lowest with the other treatment groups being intermediate (treatment effect, $p < 0.01$) (Table 3).

Table 3. Mean values for blood parameters for a subset of pigs representing six different dietary treatment groups at 6, 9 and 14 days after weaning.

	Treatment[1]							Statistics		
Item	NI-C	I-C	I-AB	I-VitE	I-Qu	I-VitC-$CuSO_4$	SEM	D	T	DxT
Vitamin E, mg/L										
Day 6	1.39	1.50	1.51	2.06	1.63	1.41	0.19	0.008	<0.001	0.509
Day 9	1.34	1.26	1.52	1.91	1.48	1.32	0.15			
Day 14	1.18	1.49	1.30	2.30	1.40	1.35	0.20			
Haptoglobin, mg/ml										
Day 6	0.89	0.90	0.75	1.38	1.10	1.10	0.13	0.001	<0.001	0.758
Day 9	1.17	1.33	0.94	1.46	1.59	1.39	0.17			
Day 14	0.85	0.88	0.60	0.98	1.15	1.37	0.19			
C-reactive protein, µg/ml										
Day 6	12.64	7.07	4.18	10.26	16.71	9.36	3.47	0.025	0.016	0.552
Day 9	22.67	15.97	6.67	12.56	23.31	17.98	5.01			
Day 14	8.51	8.40	3.45	11.48	9.11	23.45	5.44			
Malondialdehyde, mmol/L										
Day 6	1.06	1.12	1.18	1.17	1.21	1.15	0.04	<0.001	0.017	0.835
Day 9	1.19	1.16	1.26	1.23	1.29	1.25	0.05			
Day 14	1.19	1.24	1.38	1.23	1.24	1.24	0.05			

	Treatment[1]							Statistics		
Item	NI-C	I-C	I-AB	I-VitE	I-Qu	I-VitC-$CuSO_4$	SEM	D	T	DxT
Total glutathione, µg/ml										
Day 6	771	779	768	799	818	825	26.11	<0.001	0.120	0.660
Day 9	835	852	883	896	918	898	34.44			
Day 14	902	1020	969	936	964	978	32.00			
Urea, mmol/L										
Day 6	2.19	2.43	2.23	2.29	2.80	2.05	0.36	0.002	0.144	0.440
Day 9	2.29	3.23	2.84	3.28	3.94	3.98	0.56			
Day 14	2.64	1.80	2.91	2.04	3.33	1.88	0.05			
Total antioxidant capacity, copper reducing equivalents										
Day 6	369	403	362	351	397	384	17.58	<0.001	0.716	0.730
Day 9	414	404	418	415	408	410	22.11			
Day 14	429	409	421	411	440	435	16.32			
PigMAP, µg/ml										
Day 6	1567	1202	933	1440	925	1054	0.13	0.689	0.384	0.242
Day 9	875	1198	974	1220	1762	1351	0.17			
Day 14	997	862	887	1171	1211	1484	0.19			

[1] NI-C = non-infection + control; I-C = infection + control; I-AB = infection + in-feed antibiotic; I- VitE = infection + 200 IU vitamin E/kg feed; I- Qu = infection + 30 mg quercetin/kg + 50 IU vitamin E; I-VitC-$CuSO_4$ = infection + 50 IU vitamin C + 175 ppm copper + 500 mg vitamin C/kg.

Application of Research

This project was a continuation of the work from project 2C-110 where it was reported that vitamin E improved feed utilization efficiency through an eicosanoid-independent pathway. Based on the results from project 2C-110, a recommendation of > 100 IU/kg supplementation with vitamin E in a weaner diet was made to decrease the severity of *E. coli*-associated infection post-weaning. The aim of the research in the current project was to evaluate quercetin and vitamin C with copper as partial replacements of vitamin E.

Data from this project confirmed that an inclusion level of 200 IU/kg vitamin E maintains plasma vitamin E around the level of 2 mg/L (there was a slight decrease from this immediately after infection). While a plasma vitamin E level of 3 mg/L has been recommended previously to maintain immune function at the time of weaning (Jensen et al., 1988), this level is very difficult to maintain through in-feed supplementation only (Wilburn et al., 2008). Project 2C-110 reported that a vitamin E plasma concentration of 2 mg/L is still of benefit to the weaner pig possibly through a reduction in oxidative stress (Kim et al., 2016). In contrast, and despite I-VitE pigs maintaining a plasma vitamin E of 2 mg/L in the current project, it did not result in an improvement in performance, antioxidant and anti-inflammatory capacities compared with the control pigs and other treatment groups. Interestingly, higher plasma levels of vitamin E did reduce the proportion of PWD expression by 50% compared with I-C pigs, but this was not statistically significant. From previous work by Kim et al. (2016), weaner pigs supplemented with vitamin E (200 IU) had a 12-16% increase in tight junction protein mRNA, and this may have been the underlying reason for a reduction in diarrhoea in the current project.

Partial replacement of vitamin E with quercetin or a combination of $CuSO_4$ and Vitamin C (replacing 150 IU of vitamin E) had no effect on the immune response under the project's experimental conditions. This is contrary to previous results reported by Zou et al. (2016) where a similar inclusion level of quercetin was used in the diet for finisher pigs during transport. Results from the current project suggest that 30 mg quercetin/kg feed and 175-ppm copper and 500 mg Vitamin C/kg of feed are not adequate alternatives to 150 IU of vitamin E in a weaner diet.

The level of PWD in the I-C treatment group (positive control) was 60%. It is well established that some pigs are more genetically susceptible to *E. coli* infections than others and this depends on the presence of receptors in the pig intestine for the fimbriae of ETEC (Jensen et al., 2006). The MUC4 gene is a marker used for predicting the genetic susceptibility to ETEC. MUC4 -/+ and MUC4 +/+ genotypes correspond to animals with receptors for the ETEC fimbriae and a MUC4-/- genotype corresponds to animals without the receptors (Jensen et al., 2006). Testing for the susceptibility to ETEC was not performed in the current project, but the expected level of susceptibility from the source farm (from previous testing) was 70%. Previous work has reported that 65% of susceptible pigs then go on to have watery diarrhoea on the first day after infection using a similar ETEC challenge model (Jensen et al., 2006). Therefore, according to these figures, the level of PWD seen in the current project was slightly higher than the expected 45%. The ETEC infection was successful in causing increases in selected measures of inflammatory and oxidative stress blood measures. A concurrent CRC project (2A-112) has reported that a Hp plasma level above 1.14mg/ml is indicative of an inflammatory response. All treatment groups achieved this with the exception of the I-AB treatment group (see further discussions

below), however it should be noted that the day 6 Hp plasma levels were also above the 1.14mg/ml cut-off for the vitamin E (200IU) treatment group and the reason for this is not known. In saying this however, the Hp and urea response in the current project was less severe than what as previously been reported using the same ETEC challenge and a similar experiment design (Kim et al., 2016). This difference was also reflected in the performance data with pigs in the current project growing and eating 50 grams per day more than what was reported by Kim et al. (2016). This lack of severe inflammatory 'challenge' could be a reason why differences between treatment groups for all measured variables were minimal.

The discrepancy between a relatively high level of PWD and a mild inflammatory response with better than expected post-weaning performance is difficult to explain. Although rotavirus was only tested for and found in one pig (during post-mortem), it is possible that the level of diarrhoea was exacerbated from an interaction between underlying rotavirus and the ETEC challenge. A similar effect was reported by Melin et al. (2004) where pigs exposed to different serotypes of *E coli.* or one serotype of *E. coli* in conjunction with rotavirus showed more incidence of PWD than just being exposed to a single serotype of *E coli.*

The reduction in plasma Hp and C-RP seen in the I-AB treatment group highlights the anti-inflammatory effect of antibiotics, which has previously been described (Niewold, 2007), however the mechanism of action of this effect could not be explained any further from the results of the current project. Despite a reduction in selected markers of inflammation in the I-AB pigs, there was still no improvement in post-weaning performance, which fits with the notion mentioned above that the inflammatory response seen under the project's experimental conditions was minimal.

Pigs treated with an in-feed antibiotic had the same level of *E. coli* shedding as pigs not infected with *E. coli*, demonstrating that the in-feed amoxicillin was effective at treating diarrhoeic pigs. Although this seems like a simple outcome, in the search for alternatives for antibiotics for prophylactic use, it is important not to lose sight that sick animals need antibiotics and they still have a place in commercial pig production. The reason for the higher MDA levels in I-AB pigs compared with the controls is not known.

Conclusion

Inoculation with ETEC, serotype O149:K91:F4, induced diarrhoea and increases in selected measures of inflammatory and oxidative stress blood measures, but not to a level of severe inflammation as measured by plasma Hp. The inclusion of vitamin E at high levels (200 IU /kg) or the addition of quercetin and a combination of copper sulphate and vitamin C as partial replacements for vitamin E did not reduce the expression of PWD, improve post-weaning performance, or reduce inflammation or oxidative stress in ETEC-challenged pigs. Nevertheless, these results do highlight the importance of the anti-inflammatory effect of antibiotics, and understanding this effect further may assist swine industries worldwide as they explore alternatives to antibiotics.

Limitations/Risks

In this project a positive (I-C) and negative (NI-C) control were included to better assess the response of various antioxidants added to a weaner diet. Unfortunately, because all the pigs were housed in the same room, 50% of the negative control pigs showed evidence of PWD suggesting that some cross contamination between treatment groups did occur. In saying this however, the NI-C pigs along with the C-

AB pigs had the lowest *E. coli* plate score, suggesting that the PWD might have been due to a pathogen(s) other than *E. coli* serogroup O149:K91:F4. The post-mortem report from the pig that died during the experiment reported the presence of rotavirus in addition to haemolytic *E. coli* in the gastrointestinal tract. This may also help to explain why the diarrhoea persisted for longer than expected. While such a result is not ideal for experimental conditions, it perhaps highlights the impact viruses can have on pigs in the immediate post-weaning period, and perhaps our understanding of their prevalence and impact on production is not so well understood.

Recommendations

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

1. Supplementation with 200 IU vitamin E or alternatives such as quercetin and a combination of copper sulphate and vitamin C may not necessarily improve performance of weaner pigs challenged with *E. coli* under certain experimental conditions.
2. Appropriate in-feed antibiotics are effective at reducing ETEC shedding.
3. Further understanding of viruses affecting pigs in the immediate post-weaning period may better help with the management of PWD.

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