Dietary manipulation of the pro-inflammatory cascade to minimise impacts on production and health indices in weaner pigs experimentally infected with an enterotoxigenic strain of *E. Coli*

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Executive Summary

The pig's immune system is vital as its proper functioning protects the pig from disease and death. When the immune system is activated, it triggers a cascade of mechanisms including production of acute-phase proteins in the liver and pro-inflammatory cytokines in many tissues, and activation of immune cells. This process alters nutrient partitioning from body protein deposition to production of immune molecules. On the other hand, immune system activation also provokes infection responses such as fever, inhibition of appetite and vomiting, which compromise the wellbeing and welfare of pigs. In particular, the inhibition of appetite limits the pig's nutrient supply and therefore its growth and synthesis of necessary immune molecules. These infection responses are caused by production of the immunosuppressive molecule prostaglandin E_2 (PGE_2), which is produced from arachidonic acid in many tissues. Therefore, reducing PGE_2 production by blocking conversion of arachidonic acid to PGE_2, which is facilitated by an enzyme called cyclooxygenase-2, will reduce infection responses and improve health, welfare and performance of weaner pigs.

A series of studies was conducted to test the hypothesis that vitamin E and a low dose of acetylsalicylic acid (aspirin), a cyclooxygenase inhibitor, will synergistically reduce production of the immunosuppressive molecule PGE_2 and hence reduce the infection responses in weaner pigs experimentally infected with an enterotoxigenic strain of *E. coli*. Experiment 1 was conducted in a research facility using an *E. coli* infection model and Experiment 2 at a commercial R&D facility to validate the findings from Experiment 1.

Experiment 1 demonstrated that a low dose of aspirin supplementation (125 ppm) significantly improved amino acid utilization efficiency (as assessed by circulating plasma urea level) and tended to decrease PGE_2 production in the liver without affecting small intestinal histology and tight junction protein mRNA expression in the jejunal epithelium, while vitamin E supplementation greater than 100 IU significantly decreased both the acute reduction of plasma vitamin E content after weaning and plasma haptoglobin content after *E. coli* infection. Experiment 2 showed that either a low dose of aspirin or supplementation of 250 IU vitamin E in diets significantly improved feed conversion ratio until week 3 post-weaning. However, overall, there was no synergistic effect of the combined supplementation of aspirin and vitamin E on performance, intestinal barrier function and immune function of weaned pigs.

It is concluded that aspirin and vitamin E supplementation independently improved feed utilization efficiency but no synergistic effect was observed on performance, intestinal barrier function and immune function of weaned pigs. Based on tissue measurements, it is conceivable that aspirin supplementation improved performance of weaned pigs by reducing inflammation-associated amino acid waste through modulation of PGE_2 biosynthesis, while vitamin E supplementation improved performance of pigs by reducing the severity of infection through an eicosanoid-independent pathway such as oxidative tissue damage due to its antioxidant property.

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1. Introduction

The pig's immune system is vital as its proper functioning protects the pig from disease and death. When the immune system is activated, it triggers a cascade of mechanisms including production of acute-phase proteins in the liver and pro-inflammatory cytokines in many tissues, and activation of immune cells. This process alters nutrient partitioning from deposition of body protein to production of immune molecules. Previous research (2H-107) addressed this issue and suggested a nutritional solution that additional sulphur amino acid supplementation can minimize immunity-associated growth depression by improving amino acid utilization efficiency.

On the other hand, immune system activation also provokes infection responses such as fever, inhibition of appetite and vomiting, which compromise the wellbeing and welfare of pigs. In particular, the inhibition of appetite limits the pig's nutrient supply and therefore its growth and synthesis of necessity immune molecules. These infection responses are caused by production of the immunosuppressive molecule prostaglandin E_2 (PGE_2), which is produced from arachidonic acid in many tissues. Therefore, reducing PGE_2 production by blocking conversion of arachidonic acid to PGE_2, which is facilitated by an enzyme called cyclooxygenase-2, will reduce infection responses and improve health, welfare and performance of weaner pigs.

A recent review of the pig innate immune system for Australian Pork Limited by Drs John Black and John Pluske (Black and Pluske, 2012) proposed that one strategy to minimise the impact of immune system activation on production and health was to negate the infection responses via use of cyclooxygenase-2 (COX-2) inhibitors. The COX-2 enzyme catalyses arachidonic acid, which is used for PGE_2 production and PGE_2 is responsible for anorexia, fever, reduced activity and increased protein catabolism. Previously, it was reported that a low dose of acetylsalicylic acid (aspirin) (125 ppm; non-steroid anti-inflammatory inhibiting both COX-1 and 2) in diets for weaner pigs significantly improved performance and reduced diarrhoea score (Xu et al., 1990).

Another nutrient that has the ability to block PGE_2 biosynthesis through prevention of lipid peroxidation is vitamin E. Vitamin E acts as an antioxidant and is an important essential nutrient for maintenance of immune function in pigs. Recently, a survey in six Norwegian commercial herds indicated that even with more than six-fold greater supplementation of vitamin E in weaner diets than the NRC recommendation (100 IU and 16 IU/kg, respectively), plasma vitamin E levels at days -1, 4, 8 and 14 post-weaning were 2.6, 1.6, 1.5 and 1.7 mg/L (Sivertsen et al., 2007). Less than 1 mg/L plasma vitamin E is considered as a deficiency and over 3 mg/L plasma vitamin E is recommended for optimal immune function in weaner pigs (Jensen et al., 1988). This significant reduction in whole body vitamin E reserves in the first few weeks after weaning is most likely caused by increased requirement for immune function at this critical period. The extent of the reduction in plasma vitamin E level at weaning is most likely greater in Australia as vitamin E levels in diets for sow and weaner pigs are significantly lower than European countries. For example, a 'typical' commercial weaner diet generally contains around 40-75 IU vitamin E/kg compared with 100 IU/kg in European diets.

Vitamin E deficiency in pigs is known to decrease markers of cell-mediated immunity such as impaired lymphocyte proliferation (Lessard et al., 1991). Vitamin E supplementation is also known to increase humoral immune responses. For example, Ellis and Vorhies (1976) reported that, compared with a control diet (without vitamin E supplementation with approximately 20 IU/kg in the control diet), additional vitamin E supplementation at 20 and 100 IU/kg increased two- and three-fold the levels of anti *E. coli* serum antibody in pigs challenged with an intramuscular injection of *E. coli* bacterin (0.5 mL of 10^9 cfu/mL, serotype O149:K88,91:H19). Also, supplementation of 300 IU/kg vitamin E in *E. coli*-challenged broilers (post-thoracic air sacs, 1.5 mL of 5 x 10^9/mL) reduced mortality from 40% to 5% (Tengerdy and Nockels, 1975). Therefore, low vitamin E status at weaning can be a predisposing factor for viral (Beck, 2007) and bacterial diseases (Ellis and Vorhies, 1976), and bacterial infection such as *E. coli* infection further reduces body vitamin E reserves (Lauridsen et al., 2011). The protective effect of vitamin E has been reported to be associated with inhibition of the biosynthesis of PGE_2 by antagonising the lipid peroxidation of arachidonic acid and limiting the entry of precursor into prostaglandin production (Likoff et al., 1981).

Interestingly, Likoff et al. (1981) demonstrated a strong synergistic effect of vitamin E (300 IU) and a low dose of aspirin (i.p. injection of 50 mg/kg body weight) on depression of PGE_2 production and mortality in *E. coli* (LD_{50})-infected broilers. In this regard, therefore, there is potential for manipulating PGE_2 production and reducing the negative impact of inflammation by simultaneously supplementing vitamin E and acetylsalicylic acid (aspirin) in weaner pigs affected by *E. coli* infection. It was hypothesized that vitamin E and a low dose acetylsalicylic acid (aspirin), a cyclooxygenase inhibitor, will synergistically reduce production of PGE_2 and hence reduce the infection responses in weaner pigs.

2. Methodology

Two experiments were conducted to examine individual and synergistic effects of vitamin E and acetylsalicylic acid on growth performance, intestinal health, infection response and PGE_2 biosynthesis in the liver and spleen.

2.1. Experiment 1: E. coli infection study

The experimental protocol used in this study was approved by the Department of Agriculture and Food Western Australia Animal Ethics committee (AEC 6-12-19). Animals were handled according to the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

A total of 192 individually-housed male weaner pigs (Landrace x Large White) weighing 6.6 ± 0.04 kg were allocated to a 2 x 3 factorial experiment with respective factors being without and with 125 ppm aspirin (acetylsalicylic acid; Bayer) and three levels of vitamin E supplementations (50, 100 and 200 IU, *dl*-α-tocopheryl acetate; DSM). The initial replications per treatment were therefore 32 individually penned pigs. A wheat, soybean meal and skim milk powder-based basal diet was formulated to contain 15.3 MJ DE/kg (10.7 MJ NE/kg) and 0.9 g standardised ileal digestible lysine/MJ DE (Tables 1 and 2).

Table 1 - Composition of the experimental diet[1].

Ingredients	g/kg
Barley	100.0
Wheat	513.3
Canola Meal	12.4
Soybean meal	100.0
Full fat soya	50.0
Blood meal	12.4
Fishmeal	47.2
Skim milk powder	100.0
Canola Oil	38.8
Lysine	4.05
Methionine	2.40
Threonine	1.64
Tryptophan	0.10
Vit/Min[2]	0.7
Limestone	7.5
Dicalcium phosphate	7.5
Salt	2.0
Calculated composition	
DE, MJ/kg	15.3
Crude protein, g/kg	210
SID lysine, g/MJ DE	0.9

[1]Aspirin and vitamin E were replaced with wheat in the respective diets.

[2]BJ Grower 1 (BioJohn Pty Ltd, Belmont, WA, Australia), provided the following nutrients (per kg of air-dry diet): vitamins: A 7000 IU, D_3 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 fed experimental diets *ad libitum* and fresh water was supplied through a bowl drinker. All pigs were orally infected with an enterotoxigenic strain of *E. coli* (ETEC, serogroup O149:K91:F4) on days 7, 8 and 9 after weaning through oral drenching of 6, 10 and 10 mL of ETEC solution containing 1.5 x 10^8 CFU/mL, respectively. The *E. coli* solution was freshly prepared on dosing day according to the method described in Heo et al. (2010).

On day 10, eight selected pigs (close to median weight) per treatment were euthanized and the remaining 24 pigs were used for performance measurement for 21 days. Pigs were weighed and feed intake was recorded weekly to calculate performance indices. Expression of diarrhoea and the number of antibiotic treatments were recorded daily for 14 days after weaning. Blood samples were collected in lithium-heparin tubes from all pigs on days 0, 7, 14 and 21 to determine the treatment effect on plasma vitamin E, plasma urea, and acute phase protein over time. Additional blood samples were collected in EDTA tubes from euthanized pigs on day 10 to measure whole blood immune cell counts.

Table 2. Analysed chemical composition of the experimental diets.

Aspirin	0 ppm			125 ppm		
Vitamin E	50 IU	100 IU	200 IU	50 IU	100 IU	200 IU
Amino acids, g/kg as-fed						
Lys	15.1	14.4	15.8	14.5	14.6	14.3
Met	7.2	6.6	6.7	6.8	7.1	6.7
Cys	4.2	3.7	4.2	4.2	4.1	4.4
Thr	11.9	10.6	10.9	10.4	10.6	11.1
Ile	9.0	8.5	8.5	8.7	8.7	8.8
Leu	18.0	16.8	16.9	17.1	17.1	17.3
Val	11.0	10.2	10.1	10.3	10.3	10.2
Ala	10.1	9.4	9.5	9.6	9.6	9.7
Arg	12.7	11.8	11.9	12.0	12.0	12.1
Asp	20.1	18.5	18.9	18.8	18.9	19.4
Glu	49.8	44.8	46.8	47.2	46.9	47.8
Gly	9.9	9.5	9.6	9.5	9.4	9.6
His	6.5	5.8	5.9	6.0	5.9	6.0
Phe	11.4	10.4	10.6	10.8	10.8	10.9
Pro	19.3	19.3	18.2	19.2	17.9	16.8
Ser	12.6	11.4	11.7	11.6	11.7	12.1
Tyr	8.3	7.8	8.0	7.9	8.0	8.0
α-tocopherol, mg/kg as-fed	36	111	171	31	102	161

Pigs were monitored daily for the presence of diarrhoea. Faeces were scored daily depending on their consistency using the following criteria: 1 = well-formed faeces, firm to cut; 2 = formed faeces, soft to cut; 3 = faeces falling out of shape upon contact with surfaces and sloppy; 4 = pasty and liquid diarrhoea. Piglets were counted as having diarrhoea when the faecal consistency score was 4. For ethical reasons, pigs with diarrhoea were treated immediately with an intramuscular injection of Trisprim-480 (trimethropin 80 mg/ml, sulfadiazine, 400 mg/mL; Troy Laboratories, Smithfield, NSW, Australia) or Moxylan (amoxicillin 150 mg/mL, Jurox Pty Ltd., Rutherford NSW, Australia), and this was repeated daily until the diarrhoea ceased. The numbers of therapeutic antibiotic treatment were recorded for the first 14 days. Faecal beta-heamolytic *E. coli* shedding were measured on days 0, 7, 9, 11, and 13, by swabbing the rectum with a cotton bud and overnight incubation of a faecal swab at 37 °C using 5% horse blood agar plates.

2.1.1. Postmortem procedure

The eight randomly selected pigs per treatment were euthanized on day 10. Pigs were administered a single intramuscular injection of 2 mg Xylazine/kg (10 mg xylazil, Ilium Xylazil-100, Troy Laboratories Pty Ltd, Smithfield, NSW, Australia) and 5 mg Zoletil/kg body weight (10 mg tiletamine + 10 mg zolazepam, Zoletil 100, Virbac Pty Ltd, Peakhurst, NSW, Australia) to induce general anaesthesia, and then euthanized by intracardiac injection of a lethal dose (2 mL/kg) of sodium pentobarbitone solution (Lethabarb; 325 g/mL pentobarbitone sodium, Virbac Australia Pty Ltd, Peakhurst,N SW, Australia). The abdomen was then immediately opened from the sternum to the pubis, and the GIT, liver and spleen were removed. The small intestine was stripped free of its mesentery and placed on a table into sections of equal length. For measurement of villous height and crypt depth, 3–4-cm segments of the small intestine were removed at the jejunum (approximately midway along the small intestine) and ileum (5 cm cranial to the ileo-caecal junction), and carefully washed with phosphate-buffered saline (PBS)

and preserved in 10% phosphate-buffered formalin solution for subsequent histological examination. Approximately 5 g of tissue from the right lobe of the liver and spleen were collected, washed with PBS, snap frozen in liquid nitrogen and stored at -80 °C for subsequent analyses of PGE_2 and cyclooxygenase-2. Mucosal samples for tight junction protein gene expression analysis were collected from approximately 20 cm of jejunal small intestine. The jejunum was opened, washed with PBS, collected mucosal scrapings using a sterile surgical blade, and stored in RNA stabiliser to prevent RNA degradation (RNAlater, Qiagen Australia). The sample was stored at 4 °C for 24 hours and then stored at -20 °C until required for DNA extraction.

2.2. Experiment 2: Commercial validation study

Animals were handled according to the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

A total of 560 weaner pigs were allocated to a completely randomised design with four dietary treatments (4 treatments x 10 pens x 14 pigs/pen). The pens were filled in 4 batches over a 4-week period and the batch effect was included in the statistical model. The entire shed was water medicated with amoxicillin (4 days/week) as per commercial practice. The treatments were: (1) Control: base premix (80 IU vitamin E) without aspirin, (2) Aspirin: base premix with aspirin, (3) Vitamin E: base premix with 170 IU vitamin E (total vitamin E of 250 IU), and (4) Aspirin + Vitamin E: base premix + 170 IU vitamin E (total vitamin E of 250 IU) + aspirin. Diets were formulated to contain 15 MJ DE/kg and 0.8 g ileal digestible lysine per MJ DE. The composition of the experimental diets used for Experiment 2 is presented in Table 3. Owing to issues with the unregistered nature of Aspirin and potential issues with temperature stability in pelleted feeds, Aspirin was administered to treatment 2 and 4 pens via the drinking water. Aspirin was dissolved in water and dosed into the drinking water via Select Doser 640 (Think Livestock, Huntly, VIC), at a rate of 8 g Aspirin/1,000 L of drinking water in the first week and 15 g Aspirin/ 1,000 L of drinking water in subsequent weeks, to deliver the equivalent of 125 ppm of Aspirin, in-feed. Treatments 1 and 3 received plain water. There was no significant difference in water intake between treatments. The pigs were fed the experimental diets for four weeks and weekly growth rate and feed intake were measured and weekly feed conversion ratio was calculated.

2.3. Chemical analysis

Table 3. Composition of the experimental diets used for Experiment 2[1].

Ingredients, g/kg	Control	Vitamin E
Wheat	637	637
Soybean meal	76	76
Soyco meal R	12.7	12.7
Full fat soybean	24	24
Blood meal	18.7	18.7
Meat meal	66	66
Fish meal	27.3	27.3
Chocolate milk powder	100	100
Blended oil	20	20
Zinc oxide	3	3

Ingredients, g/kg	Control	Vitamin E
Choline chloride 60%	0.667	0.667
Betaine	1	1
M.H.A. Calcium[2]	0.867	0.867
Lysine-HCl	3	3
L-Threonine	1.067	1.067
Xylanase[3]	0.533	0.533
Organic acid (formic + propionic)[4]	3	3
Flavour[5]	0.2	0.2
Bentonite	0.533	0.193
Mycotoxin binder[6]	2	2
Premix[7]	2	2
Vitamin E (50%)	-	0.34
Total	1000	1000
Calculated composition, g/kg		
DE, MJ/kg	15.0	15.0
Crude protein	218	218
Calcium	10.0	10.0
digestible phosphorus	5.4	5.4
Lysine	14.1	14.1
Ileal digestible lysine/MJ DE	0.8	0.8

[1]Aspirin was delivered via water tocontrol and vitamin E diets to establish aspirinand vitamin E + aspirin treatments.
[2]Contains 84% methionine and 12% calcium, Novus International Inc., Mo. USA.
[3]Rovabio Xylan, Adisseo
[4]Biotronic SE, Biomin GmbH
[5]Pigortek 6661 Red fruits flavor, Trans Chem Pty Ltd, NSW, Australia
[6]Biofix/Mycofix plus, Biomin GmbH
[7]Provided the following nutrients (per kg of air-dry diet): vitamins: A 10,000 IU, D3 2,000 IU, E 80 mg, K 3 mg, thiamine 3 mg, riboflavin 6 mg, pyridoxine 3 mg, cyanocobalamin 20 μg, calcium pantothenate 15 mg, folic acid 1 mg, niacin 27.1 mg, biotin 100 μg. Minerals: Co 0.8 mg, Cu 20 mg, I 1.5 mg, Fe 80 mg, Mn 40 mg, Se 0.2 mg, Zn 100 mg.

Amino acid contents in the experimental diets were measured according to a method described by Ranyer (1985) with modification of Barkholt and Jensen (1989). Briefly, a 100 mg sample was hydrolysed with 6 M HCl , 0.5% phenol and 0.05% dithiodipropionic acid to convert protein-bound AA to free AA. The AA in the hydrolysate then underwent pre-column derivatisation with o-phthalaldehyde and fluorenylmethylchloroformate according to Hewlett Packard Technical Note PN 12-5966-311E. The AA derivatives were then separated and quantified by reverse phase HPLC (Hewlett Packard 1100 HPLC with Diode array detector). An Aglient Hypersill AA-ODS column (200 mm x 2.1 mm, 5 microns) with precolumn was used for all analyses. Column temperature employed was 30 ºC, detection was at 338 nm for primary and 262 nm for secondary AAs, and the flow rate was 0.3 mL/min.

Alpha tocopherol content in the feed was measured using the method of McMurray et al. (1980). Briefly 1 g of feed was homogenised in 10 ml of 6% pyrogallol by ultraturrex. One mL of 60% KOH in water was added and the sealed tubes were

heated at 70 °C for 30 min. After cooling, 5 mL of water and 20 mL of hexane was added. After extraction by vortexing, 5 ml of the hexane layer was evaporated under nitrogen and made up in 0.5 mL of methanol (0.1% butylated hydroxytoluene). The chromatographic separation was performed with an Agilent HPLC system (1100) using a Zorbax SB-C18 column (3 mm x 150 mm, 3.5 µm, Agilent). Alpha-tocopherol was quantified using fluorescence detection (ex. 296 nm and em. 330 nm).

Alpha tocopherol content in the plasma sample was analysed using the method of McMurray and Blanchflower (1979). Briefly 1 mL of plasma was deproteinised with 1 mL of 1% pryrogallol in ethanol and 5 mL of hexane was added. After extraction by vortexing, 4 mL of the hexane layer was evaporated under nitrogen and made up in 0.5 ml of methanol (0.1% butylated hydroxytoluene). The chromatographic separation was performed with an Agilent HPLC system (1100) using a Zorbax SB-C18 column (3 mm x 150 mm, 3.5 µm) (Agilent) with a methanol mobile phase. Alpha-tocopherol was quantified using fluorescence detection (ex. 296 nm and em. 330 nm).

Haptoglobin content in the plasma sample was determined using a modified method of Makimura and Suzuki (1982). Modifications are a higher concentration of sodium dihydrogen phosphate dihydrate (30 mM in reaction mix) and the use of a commercial supply of haemoglobin (Sigma-Aldrich, H2625) to produce the haemoglobin reagent (30 g/L in normal saline). The method was adapted onto an Olympus Au400 Autoanalyser (Olympus, Tokyo, Japan).

Plasma urea content was measured using a urease kinetic method with an automatic analyser (Randox Daytona, Crumlin Co., Antrim, UK). Cyclooxygenase-2 (Novatein Biosciences, Cambridge, MA, USA) and PGE_2 (R&D Systems Inc., Minneapolis, MN, USA) contents in the liver and spleen samples were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Whole blood immune cell count was done using an automatic hematology analyzer (ADIVA 2120, Bayer Healthcare, Siemens, Germany).

For mucosal histology examination, ring-shaped sections of the intestine from the jejunum and ileum were excised, dehydrated, and embedded in paraffin wax, after fixation for several days in 10% phosphate-buffered formalin. From each of these, six transverse sections (4–6 mm) were cut, stained with haematoxylin and eosin, and mounted on glass slides. The height of 10 well oriented villi, their associated crypts, and thickness of the muscular layer were measured with a light microscope (OLYMPUS CX31, Tokyo, Japan) using a calibrated eyepiece graticule.

Expressions of the mRNA encoding tight junction proteins, Zonula Occludin-1 (ZO-1) and occludin, in the jejunal mucosal scrapings were determined by a reverse transcription-polymerase chain reaction (RT-PCR). For RNA extraction, approximately 100 mg of mucosal tissue scraping from the jejunum was placed into 1mL of TRIzol Reagent (Invitrogen, VIC, Australia). This was then homogenised using a tissue homogenizer for 45 seconds. Total RNA was extracted using the PureLink RNA mini kit (Invitrogen, VIC, Australia) according to the manufacturer's instructions. Any possible contamination of genomic DNA was eliminated using PureLink DNase treatment (Invitrogen, VIC, Australia).

Table 3. Primer sequences and conditions used for real-time PCR

Primer	Sequence	Product size (bp)	Sequence Genbank ID	Concentration (nM)[a]
Occludin				
Forward	5'-GCAGCAGTGGTAACTTGGA-3'	113	NM_001163647.2	200
Reverse	5'-GTCGTGTAGTCTGTCTCGTAATG-3'			
ZO-1				
Forward	5'-CGGCGAAGGTAATTCAGTGT-3'	109	XM_003353439.2	200
Reverse	5'-CGGTTTGGTGGTCTGTAAGT-3'			
Actin ß				
Forward	5'-GCCCGTCCATCGTCCACCG-3'	127	XM_003357928	200
Reverse	5'-CAGGAGGCTGGCATGAGGTGTG-3'			

[a] Final concentration of primer used in real-time PCR.

The RNA was reverse transcribed in a 50 μl final volume using Superscript III (RT-SSIII) reverse transcriptase (100 U, Invitrogen, CA, USA) in 5 × RT buffer, with 2.5 ng/μl random primers, 10 mM each deoxynucleoside-triphosphate (dNTP's), 0.1 M dithiotreitol (DTT) and 20 U RNAsin. A heat start was applied for 2 min at 50 °C and then the RT-SSIII was added. Samples were incubated at 45 °C for 50 min and then 55 °C for 30 min. The RT enzyme was heat inactivated (90 °C for 5 min). Real-time PCR was performed using a Corbett Rotor-Gene 3000 Real Time Thermal Cycler (Corbett Research, NSW, Australia). The reactions were performed in the presence of conventional forward and reverse primers and SYBR Green (Invitrogen, VIC, Australia). Primers for the target gene's occludin and ZO-1 were designed using Primer Quest (www.idtdna.com/scitools/applications/primerquest) and then further tested using Amplify 3X. Sequences were searched and selected from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and then primers were designed using the Primer Quest option once the relevant sequence was found (Table 3). Primers were ordered from Sigma. Expression of occludin and ZO-1 were normalised to an endogenous control gene (Actin, ß) to give a ΔCt value. This accounted for variability in the initial starting amount of cDNA. An aliquot of a previously run sample from a standard curve with a known Ct value was also placed in every run, to compare run-to-run variance and to determine the amount of the gene. Cycling conditions for RT-PCR consisted of two holds of 50 °C for 2 min, 95 °C for 10 min and then cycling for 40 cycles for 95 °C 15 sec, 60 °C for 1 min and 60 °C for 60 sec. Each sample was run in triplicate.

The ΔCt has been traditionally used in Real-Time PCR data analysis. This does not take into account the efficiency of the primers that can vary from run to run. Thus we used both methods of data analysis. The actin beta Ct values were subtracted from the Ct value for each gene to give ΔCt values. These values were used to carry out statistical comparisons between and within gene's. For graphical representation, the fold variation was then determined using the $2^{-(\Delta\Delta Ct)}$ method according to publisher's protocols and the manufacture's recommendations. Fold variation was calculated by determining the difference in ΔCt values between a

chosen reference and test sample ($\Delta\Delta$Ct value), and application of the $2^{-(\Delta\Delta Ct)}$ formula. When comparing individual genes between animals, the reference used was the expression levels of each gene in the control animals.

The Pfaffl method is the relative expression of a target gene that is calculated based on the efficiency (E) of the primers used and the Ct deviation of the unknown gene versus a control, and expressed in comparison to a reference gene (Pfaffl, 2001). Individual primers were run a number of times and the efficiency calculated. This was averaged for the genes giving an average efficiency. These were actin beta 94%, occludin 105% and ZO-1 93%.

$$\text{Pfaffl ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control - sample)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control - sample)}}}$$

2.4. Statistical analysis

Experiment 1 data were analysed by two-way analysis of variance. As there was a significant batch effect, batch was used as a covariate for all statistical analysis. Plasma urea, haptoglobin and vitamin E contents measured at the start of experiment (0 d) were used as covariates for statistical analysis on subsequent measurement days. Pearson's correlation study was conducted to detect relationships between dietary treatments, production of COX-2 and PGE_2 in the liver, expression of tight junction protein genes in the jejunal epithelium, and proportion of white blood cells. Experiment 2 data were analysed by one-way analysis of variance. As there was a significant batch effect, batch was used as a covariate for statistical analysis. All statistical analysis was conducted using Genstat 15th edition (VSN International Ltd, Hemel Hempstead, UK).

3. Outcomes

3.1. Experiment 1

3.1.1. Performance and indices of post-weaning colibacillosis (PWC)

Performance response of pigs and indices of post-weaning colibacillosis (PWC) after feeding aspirin- and vitamin E-supplemented diets are presented in Table 4. Supplementation of aspirin alone improved ($P<0.05$) average daily gain (ADG) and feed conversion ratio (FCR). Significant interactions ($P<0.05$) occurred between aspirin and vitamin E for indices of PWC, namely the diarrhea index (DI) and the number of antibiotic treatments, such that supplementation of 200 IU vitamin E decreased DI and the number of antibiotic treatments only with concurrent supplementation of 125 ppm aspirin. Aspirin supplementation and 100 ppm vitamin E supplementation independently decreased ($P<0.05$) the β-haemolytic *E. coli* score.

Table 4. Interaction means for the effects of aspirin and vitamin E on performance and indices of PWC measured for 14 days after weaning in *E. coli*-infected pigs.

Aspirin (A)	0 ppm			125 ppm				*Significance*		
Vit E (E), IU	50	100	200	50	100	200	SEM	Aspirin	Vit E	A x E
ADG (g^1)	176	153	140	177	178	169	10.6	0.034	NS	NS
VFI (g^1)	195	174	167	183	185	167	10.0	NS	0.090	NS
FCR (g/g^1)	1.39	1.31	1.47	1.17	1.14	1.08	0.14	0.022	NS	NS

Aspirin (A)	0 ppm			125 ppm				*Significance*		
Vit E (E), IU	50	100	200	50	100	200	SEM	Aspirin	Vit E	A x E
DI (%[2])	6.7 ab	6.5 ab	11.4 bc	7.6[a] b	6.2[a] b	4.2[a]	1.72	NS	NS	0.043
Antibiotic[3]	1.5 ab	1.7 ab	2.3[b]	2.2[b]	1.9[a] b	1.1[a]	0.36	NS	NS	0.023
E. coli score[4]	3.5 b	2.2 ab	4.2[b]	3.0[b]	1.5[a]	2.5[a] b	0.45	0.009	0.002	NS

All pigs were experimentally infected with an enterotoxigenic strain of E. coli on day 7, 8 and 9.

[1]ADG: average daily gain; VFI: voluntary feed intake; FCR: feed conversion ratio.

[2]DI: Diarrhoea index (%); mean proportion of days with diarrhoea with respect to 14 d after weaning.

[3]Mean numbers of antibiotic treatments.

[4]Mean cumulative *E. coli* score per diet in the 14 d after weaning.

[a,b,c] Means in a row not having the same superscript are significantly different (P<0.05).

3.1.2. Plasma vitamin E, urea, haptoglobin and blood cell counts

Increasing dietary vitamin E contents significantly decreased the acute reduction of plasma vitamin E content after weaning ($P < 0.001$) on all measured days (Figure 1).

Increasing vitamin E supplementation from 50 IU to 100 IU or 200 IU significantly decreased plasma haptoglobin content after *E. coli* infection (2.51, 2.26, and 1.99 mg/mL, respectively on d 10, $P < 0.05$; 2.36, 1.8, and 1.78 mg/L, respectively on d 14, $P < 0.001$, Figure 2), while aspirin supplementation had no effect ($P > 0.05$) on plasma haptoglobin content.

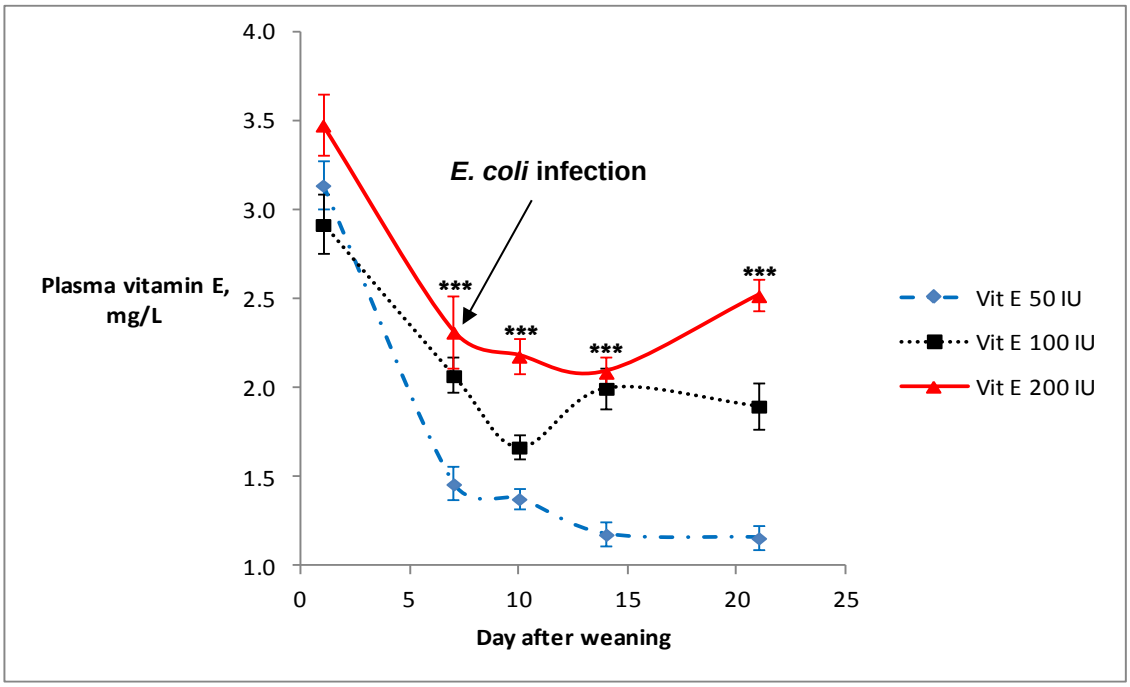

Figure 1- Effect of dietary vitamin E on plasma vitamin E contents after weaning. All pigs were experimentally infected with an enterotoxigenic strain of *E. coli* on day 7 (after blood sampling), 8 and 9.

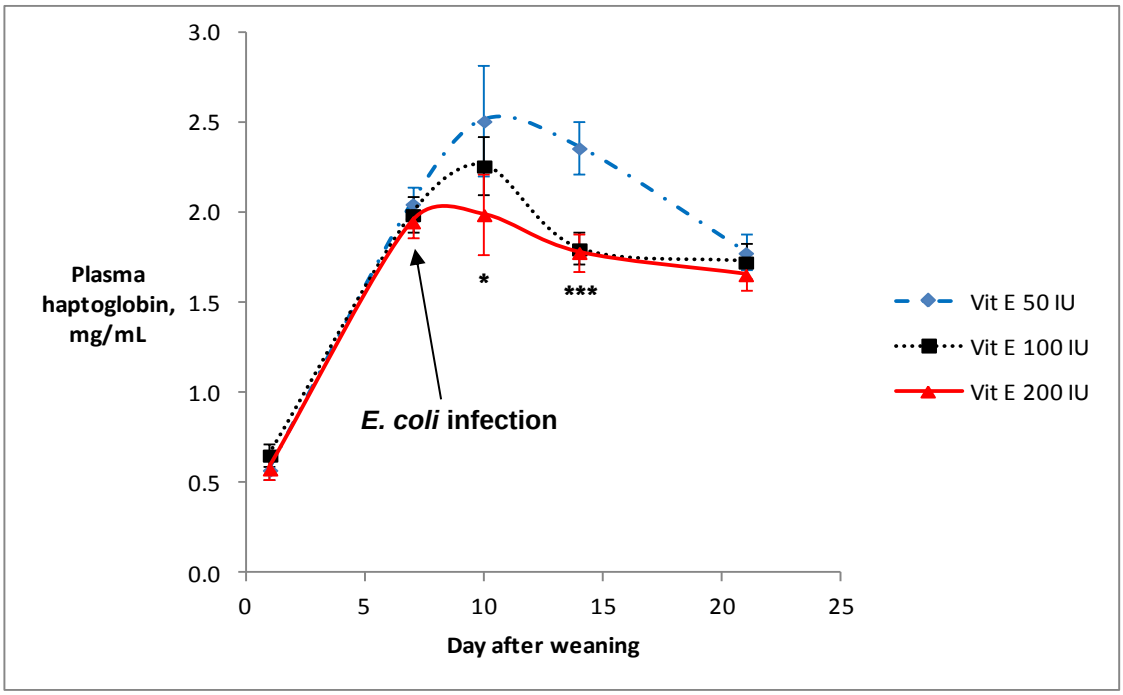

Figure 2 - Effect of dietary vitamin E on plasma haptoglobin contents after weaning. All pigs were experimentally infected with an enterotoxigenic strain of *E. coli* on day 7 (after blood sampling), 8 and 9.

A low dose of aspirin supplementation significantly decreased plasma urea content after oral *E. coli* infection (5.0 vs. 4.3 mmol/L on d 10, $P < 0.001$; 5.1 vs. 4.7 mmol/L on d 14, $P = 0.072$), while vitamin E supplementation had no effect on plasma urea content.

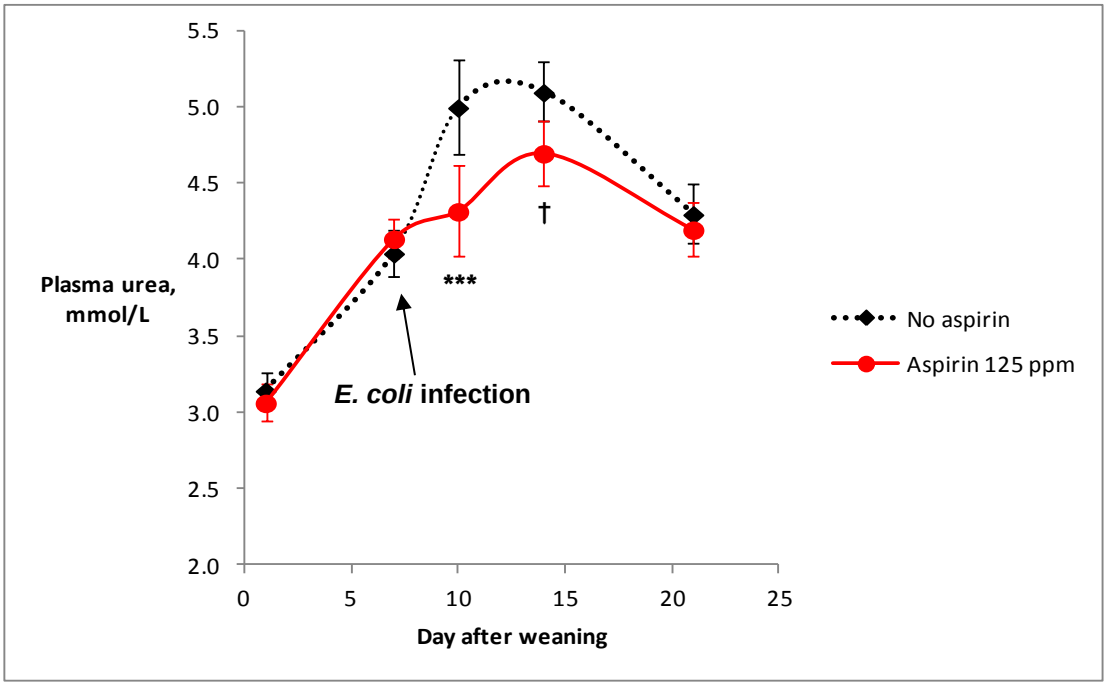

Figure 3 - Effect of a low dose of aspirin (125 ppm) on plasma urea contents after weaning. All pigs were experimentally infected with an enterotoxigenic strain of *E. coli* on day 7 (after blood sampling), 8 and 9.

Whole blood cell count results are presented in Table 5. Increasing dietary vitamin E contents from 50 to 100 and 200 IU increased the number of platelets without aspirin supplementation (447, 488, and 569 x 10^9/L, respectively), but decreased the number of platelets with aspirin supplementation (549, 598, and 435 x 10^9/L, respectively; interaction P = 0.058). Regardless of aspirin supplementation, vitamin E supplementation at 100 IU significantly increased white blood cell numbers, and increased the proportion of neutrophils while decreasing the proportion of lymphocytes (19.4 x 10^9/L vs. 17.6 x 10^9/L, P = 0.017).

Table 5 - Effect of supplementation of aspirin and vitamin E on selected blood cell numbers measured on day 10 post-weaning after experimental infection with an enterotoxigenic stain of *E. coli*.

Aspirin, ppm	0			125				*P*=		
Vit E, IU	50	100	200	50	100	200	SEM	Asp	VE	A x E
RBC, x 10^{12}/L	6.5	6.5	6.2	6.5	6.5	6.4	0.18	NS	NS	NS
CHCM, g/L	270	271	270	273	271	279	2.5	0.077	NS	NS
Platelet, x10^9/L	447	488	569	549	598	435	55.8	NS	NS	0.058
WBC, x 10^9/L	16.4	21.2	15.4	17.1	23.5	17.0	2.25	NS	0.017	NS
Neutrophil, %[1]	45.7	52.2	48.6	47.9	56.2	45.2	3.62	NS	0.078	NS
Lymphocyte, %[1]	47.6	41.7	45.4	46.5	38.3	48.2	3.53	NS	0.093	NS

RBC: Red blood cell, CHCM: mean red blood cell hemoglobin content, WBC: white blood cell.

[1]Expressed as a proportion of the white blood cell.

3.1.3. Small intestinal structure, tight junction protein mRNA expression in the jejunum, and PGE_2 and COX-2 concentration in the liver and spleen

Small intestinal structure measured at the jejunum and ileum is presented in Table 6. There was a trend that supplementation of vitamin E increased crypt depth in the ileum (P=0.089), but other than that aspirin and vitamin E supplementation had no effect on small intestinal structure.

Table 6 - Effect of supplementation of aspirin and vitamin E on small intestinal structure.

Aspirin	0 ppm			125 ppm				*P*=		
Vit E, IU	50	100	200	50	100	200	SEM	Asp	VE	A x E
Jejunum[1]										
VH, μm	407	462	434	412	415	418	37.3	NS	NS	NS
CD, μm	193	194	203	215	188	170	21.3	NS	NS	NS
VH:CD	2.4	2. 6	2.5	2.1	2.8	3.3	0.57	NS	NS	NS
MLT, μm	234	274	222	212	216	222	19.7	NS	NS	NS
Ileum[1]										
VH, μm	310	323	275	275	252	285	24.1	NS	NS	NS
CD, μm	205	295	218	198	224	229	25.7	NS	0.089	NS
VH:CD	1.6	1.2	1.3	1.6	1.2	1.4	0.20	NS	NS	NS
MLT, μm	374	393	358	333	342	349	49.3	NS	NS	NS

[1]VH: Villous height, CD: crypt depth, VH:CD: villous height:crypt depth ratio, MLT: muscular layer thickness.

Relative expression of mRNA for tight junction proteins, occludin and ZO-1, measured in the jejunal epithelium is presented in Table 7. Although, there were numerical differences towards increased tight junction protein mRNA expression with increased vitamin E supplementation without aspirin, there was a huge within-treatment variation that limited detection of statistical differences between treatments.

Table 7 - Effect of supplementation of aspirin and vitamin E on relative mRNA expression[1] of tight junction proteins in the jejunal epithelium.

Aspirin, ppm	0			125				*P*=		
Vit E, IU	50	100	200	50	100	200	SEM	Asp	VE	Asp x VE
Occludin	1.00	0.99	1.12	1.08	0.87	0.98	0.122	NS	NS	NS
ZO-1[2]	1.00	1.17	1.16	1.06	1.00	1.18	0.153	NS	NS	NS

[1]Expressed as a fold difference in relation to no aspirin and 20 IU vitamin E treatment.
[2]Zonula occludin-1

Production of PGE_2 and COX-2 were measured in the snap-frozen liver and spleen samples and the results are presented in Table 8. A low dose of aspirin tended to decrease PGE_2 production in the liver (24.5 ng/g vs. 28.2 ng/g wet tissue, P = 0.085). However, COX-2 contents in the liver were low and showed no statistical differences between treatments.

Table 8 - Effect of supplementation of aspirin and vitamin E on prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2) concentration in the liver and spleen.

Aspirin, ppm	0			125				*P*=		
Vit E, IU	50	100	200	50	100	200	SEM	Asp	VE	A x E
Liver										
PGE_2, ng/g wet tissue	30.2	27.8	26.5	27.6	22.2	23.8	3.55	0.085	NS	NS
COX-2, u/g wet tissue	0.13	0.12	0.13	0.12	0.13	0.12	0.020	NS	NS	NS
Spleen[1]										
PGE_2, ng/g wet tissue	363	317	357	261	257	377	46.8	NS	NS	NS

[1]COX-2 content could not be determined in the spleen as high hemoglobin content in the spleen interfered with the sandwich ELISA assay.

3.1.4. Relationships between treatments and measured variables

Pearson's correlation analyses were conducted to understand relationships between dietary treatments, production of COX-2 and PGE_2 in the liver, expression of tight junction protein genes in the jejunal epithelium, and the proportion of white blood cells (Table 9). Aspirin intake was negatively correlated with PGE_2 production in the liver ($P<0.05$). Cyclooxygenase-2 concentration in the liver was negatively correlated with tight junction protein gene expression in the jejunal epithelium (occludin $P<0.05$ and ZO-1 $P<0.001$). Prostaglandin E_2 concentration in the liver tend to positively correlate with proportion of lymphocytes and neutrophils ($P<0.10$).

Table 9 - Correlation coefficients between treatments and measured variables.

	Aspirin intake[1]	Vitamin E intake[1]	Liver COX-2	Liver PGE_2	Occludin	ZO-1	%Lymphocyte
Vitamin E intake	-0.090						
Liver COX-2	0.061	-0.096					
Liver PGE_2	-0.297*	-0.195	-0.141				
Occludin	0.037	-0.070	-0.357*	0.219			
ZO-1	-0.059	0.062	-0.460***	-0.092	0.422**		
%Lymphocyte	0.026	0.047	-0.158	0.263†	0.231	0.062	
%Neutrophil	-0.012	-0.052	0.137	0.278†	0.210	-0.031	-0.989***

Significance †P<0.10, *P<0.05, **P<0.01, ***P<0.001

[1]Aspirin and vitamin E intakes were calculated by multiplying individual daily feed intake and dietary concentration of aspirin and vitamin E.

3.2. Experiment 2 commercial validation

Performance responses of weaner pigs fed either aspirin (125 ppm equivalent) or vitamin E supplemented diets in a commercial farm are presented in Table 9. Individual supplementation of either aspirin or vitamin E significantly improved feed conversion ratio (P<0.05). However, there were no synergistic effects of the combined supplementation of aspirin and vitamin E on feed conversion ratio over individual supplementation.

Table 9 - Effect of individual or combined supplementation of aspirin (125 ppm equivalent) and vitamin E (250 IU) on performance of weaner pigs in a commercial weaner facility

	Control	Aspirin	Vit E	Asp + Vit E	SEM	P=
Entry Wt	5.5	5.6	5.5	5.6	0.18	NS
Week 4 Wt	12.1	12.5	12.3	12.5	0.30	NS
Wk 1-2 ADG	139	149	155	150	5.7	NS
Wk 1-2 ADFI	213	216	217	216	6.5	NS
Wk 1-2 FCR	1.74[a]	1.55[b]	1.48[b]	1.55[b]	0.065	0.044
Wk 1-3 ADG	187	196	200	200	6.4	NS
Wk 1-3 ADFI	291	297	292	294	8.1	NS
Wk 1-3 FCR	1.69[a]	1.56[b]	1.50[b]	1.53[b]	0.043	0.018
Wk 1-4 ADG	238	245	244	249	5.9	NS
Wk 1-4 ADFI	371	379	373	377	9.7	NS
Wk 1-4 FCR	1.66[a]	1.58[ab]	1.55[b]	1.55[b]	0.038	0.140

Values are means of 10 replication pens with 14 pigs per pen.
The entire shed was water medicated with amoxicillin (4 days/week) as per commercial practice.
[a,b,c] Means in a row not having the same superscript are significantly different (P<0.05).

4. Application of Research

A series of studies, including a commercial validation study, were conducted to test the hypothesis that vitamin E supplementation and a low dose of acetylsalicylic acid (aspirin), a cyclooxygenase inhibitor, will synergistically reduce production of PGE_2 and hence reduce the infection responses in weaner pigs experimentally infected with an enterotoxigenic strain of *E. coli*.

It has been reported that under weaning stress and experimental ETEC challenge, plasma vitamin E concentration is rapidly decreased to less than 1.5 mg/L when weaner pigs were fed a diet containing less than 100 IU vitamin E/kg diet (Sivertsen et al., 2007; Lauridsen et al., 2011). Considering the anti-oxidative properties of vitamin E, its requirement would be expected to be greater when oxidative tissue damage is increased in the immediate post-weaning period, due either to stress and/(or) (sub)clinical infection as a result of the significant pathogen loads in commercial production systems. Therefore, the first result we examined was whether weaning and ETEC infection decreased plasma vitamin E concentration and whether feeding a vitamin E-supplemented diet can minimize the reduction in plasma vitamin E concentration. Results from Experiment 1 indicate that 200 IU vitamin E/kg is required to maintain a plasma vitamin E level to above 2 mg/L, which is in agreement with the previous report (Wilburn et al., 2008). Although supplementation of 200 IU vitamin E was insufficient to maintain a plasma vitamin E level of 3 mg/L, which was suggested as a required level for proper immune function (Jensen et al., 1988), 200 IU vitamin E supplementation was able to maintain plasma vitamin E level to greater than 2 mg/L even after ETEC infection and also was able to return the plasma vitamin E level close to the pre-infection level by day 21 post-weaning. Wilburn et al. (2008) reported that a plasma vitamin E level of 3 mg/L was not sustainable by supplementation of 300 IU vitamin E alone, but was able to be achieved by concurrent supplementation of 300 IU vitamin E in the feed and 100 IU/L vitamin E in the drinking water.

The next question we examined was whether the increased plasma vitamin E concentration upon feeding a vitamin E-supplemented diet could improve indices for immune functions, intestinal barrier function, and infection response through modulation of COX-2 activity and PGE_2 production. Immune system activation influences growth performance of pigs through stimulation of the central nervous system by eicosanoid mediators such as PGE_2 (Rivest, 2010, Kalinski, 2012). Recognition of pathogens and/or stimulation of mast cells increase production of eicosanoid mediators, which are causative for fever due to increased metabolic rate and anorexia due to reduced appetite (Rakhshandeh and de Lange, 2011). Production of eicosanoid mediators in the cell and nuclear membranes is initiated through the action of phospholipase A_2 or C, which respectively convert phospholipid and diacylglycerol to arachidonic acid. Arachidonic acid is then converted to PGE_2 by COX-2 (Rivest, 2010, Kalinski, 2012). Unlike steroids and non-steroid anti-inflammatory drugs (NSAIDs), manipulation of dietary nutrients may not directly inhibit the activities of phospholipase or cyclooxygenase. However, it was anticipated that enhancing intestinal barrier function and protection of cell damage through supplementation of anti-oxidative vitamin E will eventually reduce production of eicosanoid mediators via negative feedback mechanisms and therefore will improve the health and growth efficiency of pigs.

Results of the present study (Experiment 1) showed that vitamin E supplementation significantly decreased the production of an acute-phase protein (haptoglobin) after ETEC infection and tended to increase the rate of cell proliferation in the ileum (increased crypt depth). Vitamin E supplementation (200

IU/kg) without aspirin supplementation increased tight junction protein mRNA expression in the jejunal epithelium by 12–16%, however, the large between-animal variation (CV 45%) hindered statistical significance. Moreover, vitamin E supplementation did not influence COX-2 activity and PGE_2 production in the liver, although again there was a numerical decrease (CV 26.9%) in PGE_2 concentration in pigs fed a vitamin E-supplemented diet. These findings indicate that vitamin E supplementation reduces the acute-phase infection response mainly by preventing oxidative cell damage under stress and ETEC infection, but has limited effect on preventing the progression of the inflammatory cascade via modulation of COX-2 activity and PGE_2 production.

Use of aspirin in pigs is not approved by the Australian Pesticides and Veterinary Medicine Authority and the Food and Drug Administration Center for Veterinary Medicine in the United States of America, but is approved in Europe (Vilalta et al., 2012). Aspirin is known to directly inhibit COX-1 and COX-2 via acetylating serine residues in the active site of the enzymes, and hence eventually inhibits production of PGE_2. Aspirin also is known to interfere with blood clotting and wound healing by reducing thromboxanes A_2 in the platelets and inhibiting platelet aggregation (Tohgi et al., 1992). However, it was reported that a low dose of aspirin (125 ppm) in a weaner pig study decreased diarrhoea and improved growth performance, and supplementation of 250 ppm aspirin did not further improve performance and in health of weaned pigs (Xu et al., 1990). Therefore, it was hypothesized that a low dose of aspirin will decrease COX-2 and PGE_2 production in the liver without affecting intestinal barrier function.

Results (Experiment 1) partly supported this hypothesis as 125 ppm aspirin tended ($P = 0.085$) to decrease PGE_2 production in the liver without affecting small intestinal histology and tight junction protein mRNA expression in the jejunal epithelium. Pearson's correlation analysis also confirmed that individual pig aspirin intake negatively correlated to PGE_2 concentration in the liver. However, aspirin supplementation did not reduce COX-2 activity in the liver, and this may be attributed to aspirin's ability to primarily inhibit COX-1 than COX-2 activity (Vilalta et al., 2012). Weaning stress itself significantly increased plasma urea concentration from 3 mmol/L on the day of weaning to 4 mmol/L on day 7 post-weaning, and experimental ETEC infection on days 7-9 further increased plasma urea concentration from 4 mmol/L to the level greater than 5 mmol/L on days 10 and 14 post-weaning. However, pigs fed an aspirin-supplemented diet maintained plasma urea content to the pre-infection level, especially immediately after ETEC infection. These results indicate that aspirin supplementation improved amino acid utilization efficiency by inhibiting progression of inflammatory cascades through reducing biosynthesis of immunosuppressive molecule PGE_2.

As the underlying mechanisms for vitamin E and aspirin on infection response of pigs are different, a synergistic reduction of infection response was hypothesized in pigs fed a diet supplemented with both vitamin E and aspirin. For example, it was reported that reducing bacterial load by supplementation of an antibiotic (doxycycline) and reducing PGE_2 production by supplementation of aspirin synergistically decreased rectal temperature in finisher pigs with respiratory disease (Vilalta et al., 2012). Moreover, Likoff et al. (1981) demonstrated a strong synergistic effect of vitamin E (300 IU) and a low dose of aspirin (i.p. injection of 50 mg/kg body weight) on depression of PGE_2 production and mortality in *E. coli* (LD_{50})-infected broilers. However, such a synergistic effect was not observed in these experiments except interaction terms observed for the DI and antibiotic treatments, where concurrent supplementation of aspirin and vitamin E was more effective than individual supplementation in Experiment 1. Although only aspirin

supplementation improved FCR in Experiment 1, a commercial validation study with a larger number of replications and antibiotic treatment (Experiment 2) clearly showed that vitamin E supplementation equally improved FCR as aspirin supplementation. Based on the results of tissue measurements, therefore, it is likely that aspirin and vitamin E supplementation independently improved performance of weaned pigs by reducing inflammation-associated amino acid waste through modulation of PGE_2 biosynthesis and by reducing the severity of infection through an eicosanoid-independent pathway such as oxidative tissue damage due to its antioxidant property.

5. Conclusions

Individual and synergistic effects of aspirin and vitamin E supplementation on performance, intestinal health and immune function were investigated in ETEC-challenged weaned pigs. Aspirin and vitamin E supplementation independently improved feed utilization efficiency but no synergistic effect was observed in performance, intestinal health and immune function of weaned pigs. Based on tissue measurements, it is conceivable that aspirin supplementation improved performance of weaned pigs by reducing inflammation-associated amino acid waste through modulation of PGE_2 biosynthesis, while vitamin E supplementation improved performance of pigs by reducing the severity of infection through an eicosanoid-independent pathway such as oxidative tissue damage due to its antioxidant property.

6. Limitations/Risks

It is likely that the level of stress around weaning, level of bacterial loads in the individual farms, and the severity of subclinical and clinical infection may have variable impact on the performance, intestinal barrier function and immune function of pigs. Therefore, the findings reported herein needs to be interpreted accordingly based on the level of stress and infection pressure in the commercial farms for application.

Aspirin may not be registered for use in pigs in Australia and therefore, until successful registration, it may only be used in a research condition as a standard for comparison with viable nutritional strategies.

7. Recommendations

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

1. A low dose of aspirin (125 ppm), pending any successful application to the APVMA in the future, is an effective way to modulate biosynthesis of immunosuppressive molecule PGE_2 and hence to reduce the immunity-associated amino acid waste.
2. Supplementation of greater than 100 IU vitamin E is recommended to decrease the severity of *E. coli*-associated infection through an eicosanoid-independent pathway such as oxidative tissue damage due to its antioxidant property around weaning.

8. References

Black JL, Pluske JL, 2012. Review of immune system in pigs. Australian Pork Limited.

Ellis R, Vorhies MW, 1976. Effect of supplemental dietary vitamin E on serologic response of swine to an *Escherichi coli* bacterin. Journal of American Veterinary Medical Association 168:231-232.

Hoe JM, Kim JC, Hansen CF, Mullan BP, Hampson DJ, Pluske JR, 2010. Effect of dietary protein level and zinc oxide supplementation on performance responses and gastrointestinal tract characteristics in weaner pigs challenged with an enterotoxigenic strain of *Escherichia coli*. Animal Production Science 50:827-836.

Jensen M, Fossum C, Edrroth M, Hakkarainen RVJ, 1988. The effect of vitamin E in the cell-mediated immune response in pigs. Journal of Veterinary Medicine B 35:549-555.

Kalinski P, 2012. Regulation of immune responses by prostaglandin E_2. The Journal of Immunology 188:21-28.

Lauridsen C, Verstergaard E-M, Hojsgaard S, Jensen SK, Sorensen MT, 2011. Inoculation of weaned pigs with E. coli reduces depots of vitamin E. Livestock Science, 137:161-167.

Lessard M, Yang WC, Elliot GS, Rebar AH, van Vleet JF, Deslauriers N, Brisson GJ, Schults RD, 1991. Cellular immune responses in pigs fed a vitamin E- and selenium-deficient diet. Journal of Animal Science 69:1575-1582.

Likoff RO, Guptill DR, Lawrence, LM, McKay CC, Mathias MM, Nockels CF, Tengerdy RP, 1981. Vitamin E and aspirin depress prostaglandins in protection of chickens against *Escherichia coli* infection. American Journal of Clinical Nutrition 34:245-251.

Makimura S, Suzuki N, 1982. Quantitative determination of bovine serum Haptoglobin and its elevation in some inflammatory diseases Nippon juigaku zasshi. The Japanese Journal of Veterinary Science 44:15-21.

McMurray CH, Blanchflower WJ, Rice DA, 1980. Influence of extraction techniques on determination of alpha-tocopherol in animal feedstuffs. Journal of the Association of Official Analytical Chemists 63:1258-1261.

McMurray CH, Blanchflower WJ, 1979. Application of a high-performance liquid chromatographic fluorescence method for the rapid determination of alpha-tocopherol in the plasma of cattle and pigs and its comparison with direct fluorescence and high-performance liquid chromatography-ultraviolet detection methods. Journal of Chromatography 178:525-31.

NHMRC. 2013. Australian code of practice for the care and use of animals for scientific purposes. 8th ed. National Health and Medical Research Council, Canberra, ACT, Australia.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29:**2002-2007.Rakhshandeh A, de Lange CFM, 2011. Immune system stimulation in the pig: Effect on performance and implications for amino acid nutrition. In "Manipulating pig production XIII" pp.31-46, ed. Van Barneveld RJ. Australasian Pig Science Association, Werribee, Vic, Australia.

Rivest S, 2010. Interactions between the immune and neuroendocrine systems. Progress in Brain Research 181:43-45.

Sivertsen T, Vie E, Bernhoft A, Baustad B, 2007. Vitamin E and selenium plasma concentrations in weanling pigs under field conditions in Norwegian pig herds. Acta Veterinarya Scandinavica 49:1-9.

Tengerdy RP, Nockels CF, 1975. Vitamin E or vitamin A protects chickens against E. coli infection. Poultry Science 54:1292-1296.

Tohgi H, Konno S, Tamura K, Kimura B, Kawano K, 1992. Effects of low-to-high doses of aspirin on platelet aggregability and metabolites of thromboxane A2 and prostacyclin. Stroke 23:1400–1403.

Vilalta C, Alcala T, Ropez-Jimenez R, Nofrarias M, Lopez-Soria S, Espin S, Varela T, Fraile L, 2012. Clinical efficacy of acetylsalicylic acid as an adjunct to antibacterial treatment of porcine respiratory disease complex. Journal of SSwine Health and Production 20:10-16.

Wilburn EE, Mahan DC, Hill DA, Shipp TE, Tang H, 2008. An evaluation of natural (RRR-¤-tocopheryl acetate) and synthetic (all-rac-¤-tocopheryl acetate) vitamin E fortification in the diet of drinking water of weanling pigs. Journal of Animal Science 86:584-591.

Xu ZR, Kornegay ET, Sweet LA, Lindemann MD, Veit HP, Watkins BA, 1990. Effects of feeding aspirin and soybean oil to weanling pigs. Journal of Animal Science 68:1639-1647.