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Development of nutritional strategies to attenuate the impact of poor environments through down-regulation of eicosanoid mediators

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Executive Summary

Reduced efficiency of feed utilization in pigs raised in a less hygienic commercial production system, relative to performance achieved in a research facility, is a major and ongoing industry constraint significantly affecting margins over feed cost (MOFC), which is the major driver for profitability of the Australian pork industry. Understanding of the systemic response to stressors and subclinical infections on intestinal barrier function and performance, and development of subsequent nutritional strategies to suppress their impact on nutrition and health of pigs, is one of the least explored areas in pig nutrition yet could have significant positive effects on health, welfare and growth efficiency of pigs. Immune system activation influences growth performance of pigs through stimulation of the central nervous system by stimulation of pro-inflammatory cytokines and subsequent *in vivo* synthesis of eicosanoid mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). However, enhancing intestinal barrier function and protection of cell damage through supplementation of particular nutrients has potential to reduce production of eicosanoid mediators via negative feedback mechanisms and therefore will improve health and growth efficiency of pigs. Therefore, the overall aim of this project was to develop nutritional strategies that attenuate the impact of a poor environment through down-regulation of eicosanoid mediators. The proposed nutrients that have been reported to have the ability to down-regulate PGE_2 and LTB_4 synthesis were boron, vitamin E and omega-3 fatty acids (*n*-3 FA).

The hypotheses tested in this project were:

(1) Dietary supplementation of boron will improve intestinal barrier function through down-regulation of eicosanoid mediators.
(2) Supplementation of vitamin E and omega-3 fatty acid with boron will synergistically down-regulate eicosanoid mediators.

Two experiments were conducted. Experiment 1 used a total of 35 individually-housed male weaner pigs weighing 6.2 ± 0.05 kg (mean ± SEM) in a completely randomised block experiment with five dietary treatments (*n*=7).

1. Control diet (commercially formulated diet).
2. Control diet + 7.5 mg/kg boron.
3. Control diet + 7.5 mg/kg boron + vitamin E (200 IU)
4. Control diet + 7.5 mg/kg boron + 20 g/kg omega-3 fatty acids.
5. Control diet + 7.5 mg/kg boron + vitamin E + 20 g/kg omega-3 fatty acids

Pigs were orally challenged with enterotoxigenic *E. coli* on day 7, 8 and 9 after weaning and euthanised for blood and tissue sample collection on day 10 after weaning. Tissue and blood samples were used for measurements of intestinal health and immunity markers. Experiment 2 used a total of 770 finisher pigs weighing 52.2 ± 1.17 kg (mean ± SEM) with the identical treatments. Completely randomised block experiment with five dietary treatments (14 pigs per pen x 11 replicate pens per treatment x 5 treatments) conducted in a commercial finisher facility. Pigs were fed for 48 days and performance parameters were recorded.

Boron content in plasma was significantly increased by supplementation of boron (0.163 vs. 0.074 mg/L, $P<0.001$). Experimental infection with an enterotoxigenic strain of *E. coli* loosened faecal consistency (58.8 vs. 33.2, day 7-9 vs. day 0-6, respectively, $P<0.001$), however there were no differences in faecal consistency between dietary treatments. The diarrhoea index was significantly increased after infection (0.143 vs. 0.014 %, $P<0.01$) without dietary effect or an interaction between

infection and dietary treatment (P>0.05). Dietary treatment did not significantly alter the epithelial integrity in any part of the small and large intestine. Also, dietary treatment did not favourably alter (P>0.05) the PGE_2 and LTB_4 synthesis in blood and in a number of organs measured. Therefore, Experiment 1 demonstrated limited benefit of supplementing boron, vitamin E and *n*-3 FA in experimentally infected weaner pigs. Experiment 2 also demonstrated that there were no additional benefits in terms of performance and carcass characteristics of the finisher pigs with these treatments.

However, correlation analysis showed that boron intake had significant positive relationships on histology of the jejunum (r=0.479, P<0.05) and whole (r=0.469, P<0.05) and empty body weight (r=0.368, P<0.10), while it had negative correlations (r=-0.440, P<0.05) with diamine oxidase (DAO) content in the liver, which is a biomarker for tissue damage. These correlations suggest that intake of boron enhanced intestinal structure, reduced damage in the liver and increased body weight of pigs, although the effects were not detectable in the treatment-based statistical analysis. Vitamin E intake tended to positively correlate (r=0.366, P<0.10) with total glutathione content in the ileal epithelium mainly due to the antioxidant capacity of vitamin E. Vitamin E intake also had positive correlations with tight junction protein, occludin and ZO-1, mRNA expression in the colon (r=0.553, P<0.01; r=0.430, P<0.05, respectively). Omega-3 fatty acid intake was positively correlated (r=0.412, P<0.05) with whole body weight at the end of the experiment, and the jejunal villous height (r=0.498, P<0.05). Omega-3 fatty acid intake was negatively correlated (r=-0.454, P<0.05) with DAO content in the liver, indicating benefits of *n*-3 FA addition on preventing liver damage and better maintaining intestinal morphological structure.

In conclusion, although the correlation analysis showed number of positive relationships between intakes of boron, vitamin E and *n*-3 FA on intestinal structure, tight junction protein expression and empty body weight, the experimental hypotheses were not supported. Therefore, it is concluded that dietary inclusion of boron, vitamin E and *n*-3 FA in diets for weaner and finisher pigs had limited impact on intestinal health and performance of pigs. However, given the positive relationship between intake of boron, vitamin E and *n*-3 FA and some of the indicators for intestinal health, further refined research is warranted to elucidate whether concentrations of boron in the diet would have impact on intestinal health and performance of pigs.

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1. Background to Research

Reduced efficiency of feed utilization in pigs raised in a less hygienic commercial production system than a research facility is a major and ingoing industry constraint significantly affecting margins over feed cost (MOFC), which is the major driver for profitability of the Australian pork industry. Although there are many factors affecting MOFC, an important factor is the reduced growth rate and feed utilization efficiency caused by subclinical infections and socio-environmental stresses such as social hierarchy, temperature and transportation. Understanding of the systemic responses to stressors and subclinical infections on intestinal barrier function and performance, and development of subsequent nutritional strategies to suppress their impact on nutrition and health of pigs, is one of the least explored areas in pig nutrition yet could have significant positive effects on health, welfare and growth efficiency of pigs.

Immune system activation influences growth performance of pigs through stimulation of the central nervous system by eicosanoid mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). 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. 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 either PGE_2 or LTB_4 by cyclooxygenase and lipoxygenase, respectively. In human medicine, steroids and non-steroid anti-inflammatory drugs (NSAIDs) such as aspirin are used to directly inhibit phospholipase and cyclooxygenase activities. However, the pig industry cannot rely solely on any available steroids and NSAIDs to reduce the impact of eicosanoid mediators on infection-induced anorexia, and alternative dietary strategies need to be developed to reduce metabolic waste of nutrients. Unlike steroids and NSAIDs, manipulation of dietary nutrients may not directly inhibit the activities of phospholipase, cyclooxygenase or lipoxygenase. However, enhancing intestinal barrier function and protection of cell damage through supplementation of particular nutrients will eventually reduce production of eicosanoid mediators via negative feedback mechanisms and therefore will improve health and growth efficiency of pigs. The overall aim of this project is to develop nutritional strategies that attenuate the impact of poor environment through down-regulation of eicosanoid mediators, and the following nutrients will be tested as candidate strategies.

2. Objectives of the Research Project

The overall objective of this research was to determine the effect of dietary boron, and synergistic effects of boron with vitamin E and omega-3 fatty acids, on intestinal barrier function and circulating eicosanoid mediators.

The hypotheses tested in this project were:

1) Dietary supplementation of boron will improve intestinal barrier function through down-regulation of eicosanoid mediators.
2) Supplementation of vitamin E and omega-3 fatty acid with boron will synergistically down-regulate eicosanoid mediators.

3. Introductory Technical Information

Boron is a naturally occurring non-metallic mineral which does not accumulate in the tissue, and is widely used for medical purposes due to its antibacterial properties and ability to reduce heavy metal toxicity (Turkez *et al*., 2012). In the body, most boron (> 96%) exists as boric acid and reacts with molecules with hydroxyl groups to form boron ester. Therefore, boric acid promptly forms complexes with metabolically important sugars such as ribose, which is a component of adenosine (Nielsen, 2009; Nielsen and Meacham, 2011). This chemical characteristic allows boron to react with signaling molecules containing adenosine. Apart from this, dietary boron is known to inhibit both PGE_2 and LTB_4 production by inhibiting cyclooxygenase and lipoxygenase activity in leukocytes in rats (Rajendran *et al.*, 1994, Hunt and Idso, 1999, Nielsen *et al*., 2007). Hunt and Idso (1999) suggested that dietary boron down-regulates leukocyte 6-phosphogluconate dehydrogenase and reduces reactive oxygen species (ROS) produced by neutrophils for local phagocytosis, but exacerbates the inflammatory response in excess (Hunt and Idso, 1999). Boron is also known to inhibit the activity of serine proteases such as tryptase, the proteolytic enzymes released by activated mast cells and leukocytes which degrade structural proteins of pathogens but also degrade structural proteins of the host cells (Hunt, 2003).

Only a few pig studies have been conducted with boron and the authors reported the beneficial effects of boron on performance and immune function, although no pig study has specifically measured the effects of boron on PGE_2 and LTB_4 production. Armstrong and Spears (2003) fed either a control diet or a diet supplemented with 5 mg boron/kg as sodium borate and found a significant increase in average daily gain due mainly to increased feed intake in the 49 d after weaning (0.47 versus 0.36 kg), and also during the subsequent grower phase (next 79 d, 1.00 versus 0.85 kg). More interestingly, this particular study tested a local inflammatory response by measuring skin-fold swelling after intra-dermal injection of phytohaemagglutin (150 μg) at 120 d of age and found that pigs fed a 5 mg/kg boron diet significantly reduced skin-fold swelling. Consistent improvements in daily gain, feed intake and local inflammation response were repeatedly reported in pigs fed a 5 mg boron diet (Armstrong *et al*., 2001; Armstrong and Spears, 2001; Armstrong *et al*., 2002). Given that the eicosanoid mediators (PGE_2 and LTB_4) induce anorexia which is partly responsible for decreased growth in subclinical and clinical infection, the consistent improvement in feed intake and daily gain in pigs fed a 5 mg/kg boron diet may advocate that boron reduced *in vivo* production of eicosanoids.

Some publications have reported interactive effects of dietary boron and *n*-3 fatty acids on $PGF_{2}\alpha$ production and a synergistic reduction in cell damage caused by the ROS in rats (Nielsen *et al*., 2007; Nielsen, 2009). Omega-3 fatty acids are well known to reduce production of eicosanoid mediators. One of the mechanisms is that the linolenic acids (18:3, n-3) are elongated and desaturated to produce eicosapentaenoic acids (EPAs), which are incorporated in cell and nuclear membrane phospholipids. This process is known to compete for the same enzymes (Δ6 desaturase, elongase, Δ5 desaturase) as those required for conversion of linoleic acids (*n*-6 fatty acid) to arachidonic acids, which are also incorporated in cell/nuclear membrane phospholipids and used as a precursor for production of eicosanoid mediators. Another beneficial effect of feeding *n*-3 fatty acids is that the membrane phospholipid embedded *n*-3 fatty acids (EPA) are used for production of anti-inflammatory prostaglandin families such as PGE_3 when pathogen/antigen invasions are detected, while membrane phospholipid embedded *n*-6 fatty acids (arachidonic acids) are converted to immunosuppressive PGE_2 and LTB_4 (Goddeeris *et al*., 2002). As different mechanisms are involved in the anti-inflammatory effects of boron and other suggested nutrients such as *n*-3 fatty acids, and vitamin E, there is a need to understand the nature of synergism that might exist between boron, vitamin E, and *n*-3 , which are

all known to reduce PGE_2 production possibly through different mechanisms in pigs.

Based on this background knowledge, this experiment will aim to determine the effect of dietary boron, and synergistic effects of boron with vitamin E and omega-3 fatty acids, on intestinal barrier function and circulating eicosanoid mediators.

4. Research Methodology

Two experiments were conducted to test the study hypotheses.

4.1. Experiment 1

A total of 35 individually-housed male weaner pigs were used in a completely randomised block experiment with five dietary treatments (*n*=7).

- Treatments:
  6. Control diet (commercially formulated diet).
  7. Control diet + 7.5 mg/kg boron.
  8. Control diet + 7.5 mg/kg boron + vitamin E (200 IU)
  9. Control diet + 7.5 mg/kg boron + 20 g/kg omega-3 fatty acids.
  10. Control diet + 7.5 mg/kg boron + vitamin E + 20 g/kg omega-3 fatty acids

The increased use of boron in this study (7.5 vs. 5 mg/kg) compared with a previous publication (Armstrong *et al*., 2001) was determined to compensate for lower feed intake due to weaning stress and experimental infection with haemolytic *E. coli*. The experimental protocol used in this study was approved by the Department of Agriculture and Food Western Australia Animal Ethics committee (AEC 1-14-01). 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 35 Large White x Landrace x Duroc pigs weighing 6.2 ± 0.05 kg (mean ± SEM) was sourced and transported from a commercial breeding farm with a high health status at the time of weaning (pigs approximately 21–24 days old). Upon arrival all pigs were ear tagged, weighed and stratified based on their live weight to five dietary treatments. Pigs were fed their respective diets for 10 days after weaning and live weight of pigs were recorded on days 0, 7 and 10. Feed intake was recorded on day 7 and 10 to calculate mean daily intake of boron, vitamin E and omega-3 fatty acids. Pigs were orally infected with enterotoxigenic strain of *E. coli* on days 7, 8 and 9 to stimulate their immune system. All pigs were euthanized on day 10 for collection of tissue samples. Diets were balanced to contain equivalent levels of amino acids and energy. The nutrient supply met all requirements of pigs of this age and genotype. A wheat, soybean meal and whey powder-based basal diet was formulated to contain 15.0 MJ DE/kg (10.35 MJ NE/kg) and 0.9 g standardised ileal digestible lysine/MJ DE (Tables 1 and 2).

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 9.67 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). Faecal consistency score, diarrhoea index, and the number of therapeutic antibiotic treatments were recorded for 10 days. Faecal beta-haemolytic *E. coli* shedding was measured on days 0, 5, 7, and 9. Following swabbing the rectum of each pig with a cotton bud, the swab contents were cultured overnight and beta-haemolytic *E. coli* colonies were counted on blood agar plates.

Blood samples were collected in lithium-heparin tubes from all pigs on days 7 and 10 to determine the treatment effect on plasma vitamin E, plasma urea, and acute phase protein before and after infection. Additional blood samples were collected in EDTA tubes on days 7 and 10 to measure whole blood immune cell counts.

Table 1 - Composition of the experimental diets in Experiment 1(g/kg)[1].

Treatment	Control	Boron	B+Vit E	B+n-3 FA	B+Vit E+n-3FA
Barley	100	100	100	100	100
Wheat	495	495	495	495	495
Soybean meal dehulled	150	150	150	150	150
Blood meal 85% Ring dried	20	20	20	20	20
Fishmeal 60%	83	83	83	83	83
Whey powder, acid	100	100	100	100	100
Tallow	32	32	32	12	12
Lysine	2.73	2.73	2.73	2.73	2.73
Methionine	2.29	2.29	2.29	2.29	2.29
Threonine	1.30	1.30	1.30	1.30	1.30
Tryptophan	0.13	0.13	0.13	0.13	0.13
Vi/Min Premix[2]	1	1	1	1	1
Limestone	5.24	5.24	5.24	5.24	5.24
Dicalcium Phosphate	4.45	4.45	4.45	4.45	4.45
Salt	2	2	2	2	2
Choline Chloride 60%	0.36	0.36	0.36	0.36	0.36
Boric acid[3]		0.0429	0.0429	0.0429	0.0429
Vitamin E (50%)[4]			0.4		0.4
n-3 FA[5]				20	20
Calculated nutrient composition, g/kg					
DE, MJ/kg	15.0	15.0	15.0	15.0	15.0
NE, MJ/kg	10.35	10.35	10.35	10.35	10.35
SID Lys, g/MJ DE	0.9	0.9	0.9	0.9	0.9
Crude protein	213	213	213	213	213
Crude fat	52	52	52	52	52
NDF	96	96	96	96	96
ADF	28	28	28	28	28
Ca	9.0	9.0	9.0	9.0	9.0
Faecal digestible phosphorus	4.5	4.5	4.5	4.5	4.5

[1]Boric acid and vitamin E were replaced with wheat (w/w) in the respective diets.

[2]BJ Grower PX (BioJohn Pty Ltd, Belmont, WA, Australia), provided the following nutrients (per kg of air-dry diet): vitamins: A 8000 IU, D3 1500 IU, E 70 mg, K 2 mg, thiamine 2 mg, riboflavin 5 mg, pyridoxine 2.5 mg, cyanocobalamin 15 μg, calcium pantothenate 40 mg, folic acid 2.5 mg, niacin 25 mg, biotin 100 μg. Minerals: Co 0.5 mg (as cobalt carbonate), Cu 20 mg (as copper sulphate), I 1.0 mg (as potassium iodine), Fe 60 mg (as ferrous sulphate), Mn 40 mg (as manganous oxide), Se 0.3 mg (organic 0.2, inorganic 0.1), Zn 100 mg (as zinc sulphate).

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 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 was measured on days 0, 7, and 9 by swabbing the rectum with a cotton bud and overnight incubation of a faecal swab at 37 °C using 5% horse blood agar plates.

On day 10, all pigs were euthanized (see 4.1.1), with intestinal tissue, liver, spleen and mesenteric lymph node samples collected. Villous height, crypt depth, and epithelial structure in the duodenum, jejunum and ileum were measured using standard procedures. To measure paracellular translocation of haemolytic *E. coli* into the systemic circulation, tight junction protein mRNA gene expression in the ileal epithelium was measured. Concentration of eicosanoid mediators (PGE_2 and LTB_4) was measured in plasma, liver, spleen and ileal mucosa samples using standardised ELISA techniques (see 4.3).

Table 2 - Analysed chemical composition of the experimental diets.

Treatment	Control	Boron	B+Vit E	B+n-3 FA	B+Vit E+n-3FA
Boron, mg/kg	7.4	14.5	16.5	14.0	15.5
α-tocopherol, mg/kg	55	56	239	58	301
Fatty acid profile, g/kg					
C10:0	0.00	0.00	0.00	0.02	0.01
C12:0	0.03	0.04	0.03	0.03	0.03
C14:0	0.35	0.43	0.32	0.33	0.32
C14:1n5	0.00	0.00	0.00	0.00	0.02
C15:0	0.12	0.15	0.11	0.11	0.11
C15:1	0.03	0.04	0.03	0.03	0.03
C16:0	9.40	10.81	9.06	8.15	8.33
C16:1n7	0.35	0.43	0.32	0.33	0.33
C17:0	0.19	0.21	0.17	0.17	0.17
C17:1	0.09	0.10	0.08	0.08	0.08
C18:0	2.59	2.67	2.31	2.62	2.58
C18:1cis+trans	12.4	15.2	11.7	12.1	11.3
C18:2n6	35.7	44.2	35.0	27.1	26.2
C18:3n3	4.58	6.00	4.68	18.7	18.0
C20:0	0.17	0.16	0.15	0.13	0.13
C20:1	0.22	0.29	0.22	0.21	0.20
C20:4n6	0.30	0.37	0.28	0.29	0.30
C22:0	0.16	0.15	0.13	0.12	0.11
C20:5n3	0.62	0.79	0.58	0.62	0.59
C22:4n6	0.32	0.40	0.30	0.33	0.31
C24:0	0.07	0.07	0.08	0.07	0.07
C22:5n3	0.15	0.19	0.14	0.15	0.15
C24:1	0.07	0.00	0.06	0.07	0.07
C22:6n3	3.81	4.96	3.59	3.77	3.69
Total FA	71.7	87.7	69.3	75.6	73.1
n-6 FA	36.3	45.0	35.5	27.8	26.8
n-3 FA	9.16	11.93	8.99	23.3	22.4
n-6:*n*-3 ratio	4.0	3.8	4.0	1.2	1.2
Saturated fat	13.1	14.7	12.4	11.7	11.9
Unsaturated fat	58.6	73.0	57.0	63.8	61.3

4.1.1. Post-mortem procedure

All pigs 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 duodenum (approximately 5 cm from pylorus), 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 and mid colon. The jejunum and colon were 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.

4.2. Experiment 2

A completely randomised block experiment with five dietary treatments (14 pigs per pen x 11 replicate pens per treatment x 5 treatments = 770 pigs) in a commercial finisher facility. Average starting weight was 52.2 ± 1.17 kg (mean ± SEM).

Treatments:

1. Control diet (commercially formulated diet).
2. Control diet + 7.5 mg/kg boron.
3. Control diet + 7.5 mg/kg boron + vitamin E (200 IU)
4. Control diet + 7.5 mg/kg boron + 20 g/kg omega-3 fatty acids.
5. Control diet + 7.5 mg/kg boron + vitamin E + 20 g/kg omega-3 fatty acids

Diets were formulated to contain 13.7 MJ DE/kg, and 0.60 g SID lysine/MJ DE. Composition of the experimental diets is presented in Table 3. Pigs received their respective experimental diets *ad libitum* for 48 days and fresh water was accessible via a nipple drinker. Water medication was supplied during the 19-20th days (CTC, 20 g/tonne/day) and the 33-34th days (Tylan in water, 23 g/tonne/day). Pigs were weighed and feed intake was measured at days 0, 32 and 48. At the end of the 48 days of the experiment, all pigs were slaughtered at a commercial abattoir and carcass composition was recorded.

4.3. Chemical and histological analysis

Amino acid contents in the experimental diets were measured according to a method described by Ranyer (1985) with the 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 °C, detection was at 338 nm for primary and 262 nm for secondary AAs, and the flow rate was 0.3 mL/min.

Table 3 - Composition of the experimental diet used in Experiment 2 (g/kg)[1].

Treatment	Control	Boron	B+Vit E	B+n-3 FA	B+Vit E+n-3FA
Wheat	600	600	600	600	600
Barley	170	170	170	170	170
Canola meal 36%	150	150	150	150	150
Meat meal 65%	15	15	15	15	15
Water	15	15	15	15	15
Natuphos 5000	0.01	0.01	0.01	0.01	0.01
Porzyme 9310	0.02	0.02	0.02	0.02	0.02
Semi refined fish oil	-	-	-	10	10
Tallow	20	20	20	10	10
Limestone	12.7	12.7	12.7	12.7	12.7
Dicalcium phosphate	4	4	4	4	4
Methionine	0.533	0.533	0.533	0.533	0.533
Boric acid	-	1	1	1	1
Mineral blend	1.63	1.63	1.63	1.63	1.63
Lysine	4.47	4.47	4.47	4.47	4.47
Threonine	1.45	1.45	1.45	1.45	1.45
Fysal SP	2	2	2	2	2
Cu	0.83	0.83	0.83	0.83	0.83
Vitamin E	-	-	0.8	-	0.8
Vitamin blend	0.133	0.133	0.133	0.133	0.133
Isoleucine	0.133	0.133	0.133	0.133	0.133
Salt	2	2	2	2	2
Salinomycin 120	0.5	0.5	0.5	0.5	0.5
Calculated nutrient composition, g/kg					
DE, MJ/kg	13.7	13.7	13.7	13.7	13.7
SID Lys, g/MJ DE	0.60	0.60	0.60	0.60	0.60
Crude protein	159	159	159	159	159
Crude fat	37	37	37	37	37
Crude fibre	45	45	45	45	45
Calcium	9	9	9	9	9
Faecal digestible phosphorus	4	4	4	4	4
Vitamin E	19	19	218	19	218
n-6 FA	7.7	7.7	7.7	8.7	8.7
n-3 FA	1.2	1.2	1.2	3.1	3.1
n-6:*n*-3 FA ratio	6.2	6.2	6.2	2.8	2.8

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

[2]BJ Grower PX (BioJohn Pty Ltd, Belmont, WA, Australia), provided the following nutrients (per kg of air-dry diet): vitamins: A 2667 IU, D3 533 IU, E 20 mg, K 0.4 mg, thiamine 0.6 mg, riboflavin 2.0 mg, pyridoxine 1.2 mg, cyanocobalamin 2.0 mg, calcium pantothenate 6.0 mg, niacin 8.0 mg, Minerals: Co 0.5 mg, Cu 200 mg, I 0.5 mg, Fe 70 mg, Mn 29 mg, Se 0.25 mg, Zn 52 mg, Cr 200 μg.

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).

Haptoglobin content in the plasma sample was determined using a modified method of Makimura and Suzuki (1982). Modifications were use of 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). The PGE_2 (R&D Systems Inc., Minneapolis, MN, USA) contents in the liver and ileal mucosa samples were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Whole blood immune cell count was done using an automatic haematology analyser (ADIVA 2120, Bayer Healthcare, Siemens, Germany).

Total glutathione and diamine oxidase contents in the liver and ileal epithelium were analysed using commercially available ELISA kits (total glutathione, Abnova Glutathione assay kit, Sapphire Bioscience, Redfern, NSW; diamine oxidase, Life Research, Welland, SA). Plasma content of leukotriene B_4 (LTB_4) was analysed using a leukotriene B_4 ELISA kit (Abcam Pty Ltd, Melbourne). The malondialdehyde (MDA) content in the liver and ileal epithelium was measured using the modified method of AMSA (2012) and Jo and Ahn (1998). Tissue samples were stored at - 20° C prior to analysis, but with the following modifications to the colour reagent: inclusion of sodium docecylsulphate and butylated hydroxytoluene to final concentrations of 0.42% and 0.09%, respectively. Homogenised tissue was added at the rate of 0.3 ml to 1.5 ml of colour reagent. The MDA was quantified using the standard 1,1,3,3-tetramethoxypropane with spectrofluorometric measurement (Jo and Ahn, 1998) at 510 nm excitation and 560 nm emission using a POLARstar Omega plate reader (BMG Labtech Pty. Ltd. Mornington, Victoria).

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, and COX-2 in the jejunal and colonic 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).

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, ZO-1, AND COX-2 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 4). Primers were ordered from Sigma. Expression of occludin and ZO-1 were normalised to an endogenous control gene (Actin, β) 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.

Table 4 - 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'			
COX-2				
Forward	5'-CCAGCACTTCACCCATCAGT-3'	132	Nm_214321.1	200
Reverse	5'-AGGCGCAGTTTATGCTGTCT-3'			
Actin β				
Forward	5'-GCCCGTCCATCGTCCACCG-3'	127	XM_003357928	200
Reverse	5'-CAGGAGGCTGGCATGAGGTGTG-3'			

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

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).

4.4. Statistical analysis

In Experiment 1, data were analysed by one-way ANOVA with the pig considered as the experimental unit for all data analysis. Blood measures conducted before and after infection were analysed using a repeated measure ANOVA to examine treatment effects as well as interaction between infection and treatment. 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 ANOVA. Fisher's-Protected Least Significant Difference test was conducted to separate means where significant treatment effect occurred under the ANOVA analysis. All statistical analysis was conducted using Genstat 15th edition (VSN International Ltd, Hemel Hempstead, UK).

5. Results

Experiment 1

5.1. Boron content in plasma and the liver

Boron content in plasma was significantly increased by supplementation of boron (0.163 vs. 0.074 mg/L, SE ± 0.018, P<0.001), and experimental ETEC infection significantly increased plasma boron content (0.203 vs. 0.087 mg/L, SE ± 0.0116, P<0.001, Figure 1). However there was no interaction between infection and dietary treatment on plasma boron content. Supplementation of boron significantly increased boron content in the liver when measured at 10 days after weaning (0.196 vs. 0.057, SE ± 0.0281, P<0.01).

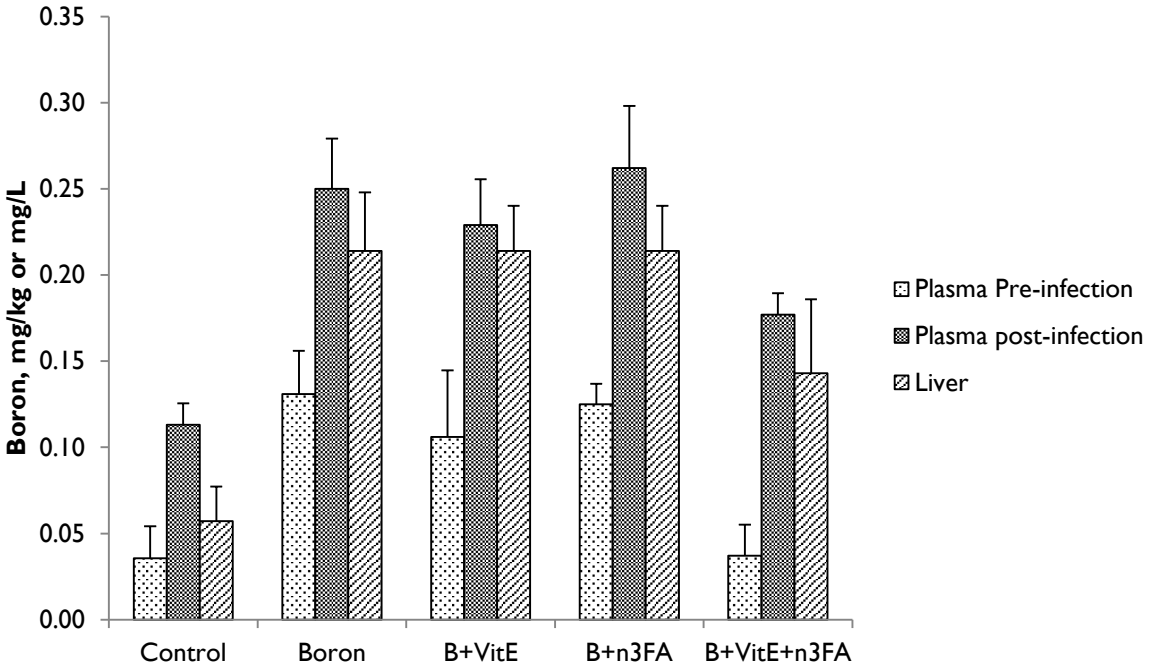

Figure 1. Boron content in plasma measured before and after experimental infection with an enterotoxigenic strain of E. coli*, and boron content in the liver at 10 days after weaning.*

5.2. Faecal consistency, faecal shedding of E. coli*, diarrhoea index and therapeutic antibiotic treatments*

Experimental infection with an enterotoxigenic strain of *E. coli* loosened faecal consistency (58.8 vs. 33.2, day 7-9 vs. day 0-6, respectively, P<0.001, Figure 2), however there were no differences in faecal consistency between dietary treatments. There was a tendency of an interaction between infection and dietary treatment such that faecal consistency increased more significantly in pigs fed the boron + *n*-3 fatty acid diet compared with pigs fed the other diets (interaction P=0.057). Mean faecal *E. coli* shedding score was increased from 0.51 to 1.91 (SE 0.253) after the experimental infection (P<0.001), with a tendency that pigs fed the boron + *n*-3 fatty acid diet shed more *E. coli* in their faeces than pigs fed the other diets (2.0 vs. <1.14, SE ±0.415, P=0.088). Diarrhoea index was significantly increased after infection (0.143 vs. 0.014 %, SE ±0.0428, P<0.01) without dietary effect or an interaction between infection and dietary treatment. Consequently, the proportion of days with therapeutic antibiotic treatments was significantly increased following experimental infection (21.4 vs. 4.5%, SE ±4.87,

P<0.05). There was an approaching trend of an interaction between infection and dietary treatment for the proportion of days with therapeutic antibiotic treatment (P=0.105) such that pigs fed the boron diet decreased therapeutic antibiotic treatment after infection from 12.2% to zero % while pigs fed the control and boron+n3-fatty acids diet increased the therapeutic antibiotic treatments, from zero % to 42.9% and 35.7% respectively. Pigs fed boron+Vit E (14.3%) and boron+Vit E+*n*-3 fatty acids (14.3%) intermediately increased the proportion of days with therapeutic antibiotic treatment after experimental infection.

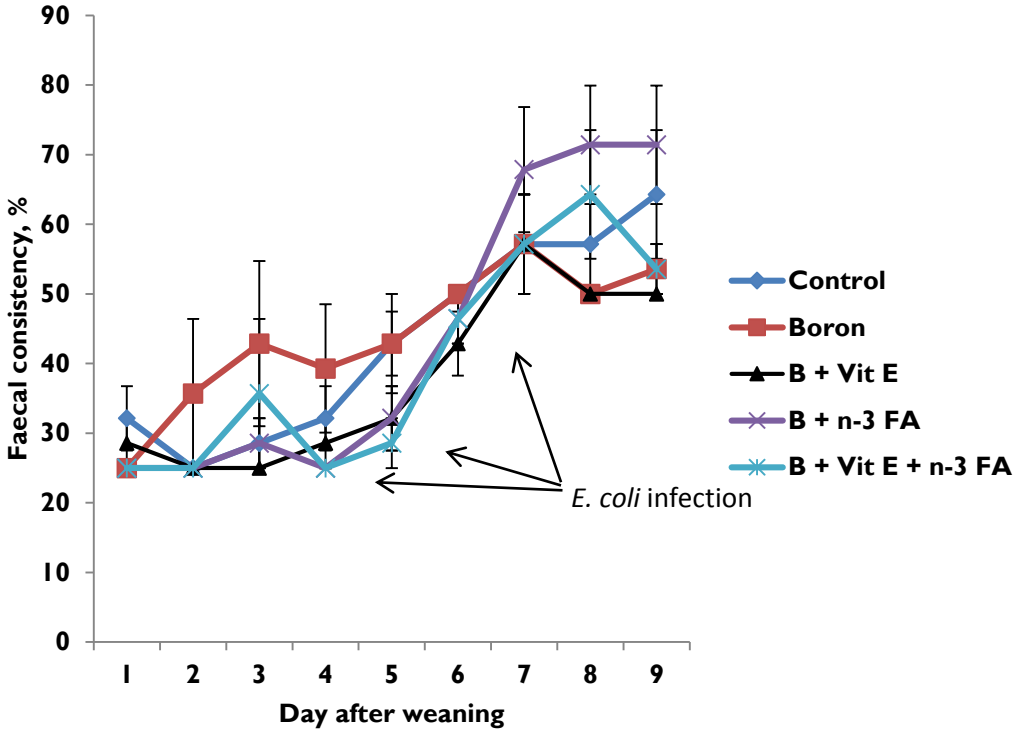

Figure 2. Faecal consistency (%) of pigs fed diets supplemented with combinations of boron, vitamin E and n-3 fatty acids before and after experimental infection with an enterotoxigenic strain of E. coli.

5.3. Live weight, empty body weight and weights of the liver and spleen

There were no treatment effects either on the live weight at day 10 post-weaning, empty body weight, and the weights of the liver and spleen in relation to the kg body weight (P>0.05, Table 5).

Table 5 - Effect of supplementation of boron alone or with omega-3 fatty acids and 200 IU vitamin E on live weight, empty body weight and the relative weight of the liver and spleen

	Control	Boron	B+Vit E	B+n3FA	B+Vit E+n3FA	SEM	Significance
Start weight, kg	6.2	6.3	6.2	6.2	6.2	0.05	0.442
Final body weight, kg	6.7	6.9	6.9	6.8	6.3	0.26	0.517
Empty body weight, kg	5.5	5.6	5.6	5.4	5.3	0.17	0.634
Liver, g/kg body weight	32.6	32.1	31.7	27.5	30.9	2.37	0.586
Spleen, g/kg body weight	3.7	3.7	5.0	4.3	4.3	0.54	0.425

5.4. Blood cell counts

Infection with ETEC between days 7 and 9 after weaning significantly increased the counts of leukocytes (P<0.001), lymphocytes (P<0.001), neutrophils (P<0.001), monocytes (P<0.001), basophils (P<0.05) and platelets (P<0.01, Table 6). Pigs fed the B+Vit E+*n*-3 fatty acids diet significantly increased their number of lymphocytes (P<0.05) and hence tended to increase the lymphocyte to neutrophil ratio (P=0.073) compared with pigs fed the other diets. Pigs fed either Boron or B+Vit E diets significantly decreased the number of monocytes compared with the pigs fed a control or B+Vit E+n-3 fatty acids diets. There were no interactions between infection and dietary treatment on all measured variables.

5.5. Plasma urea, haptoglobin and leukotriene B_4

Infection increased plasma urea content (P<0.01) while dietary treatments had no effect on plasma urea content (Table 7). Both infection and dietary treatment had no effects on plasma haptoglobin content. Plasma LTB_4 content tended to be influenced by an infection by dietary treatment interaction (P=0.063, Figure 3) such that pigs fed the control and B+Vit E+n-3 fatty acid diets decreased plasma LTB_4 content after infection, while pigs fed the other diets maintained or increased plasma LTB_4 content.

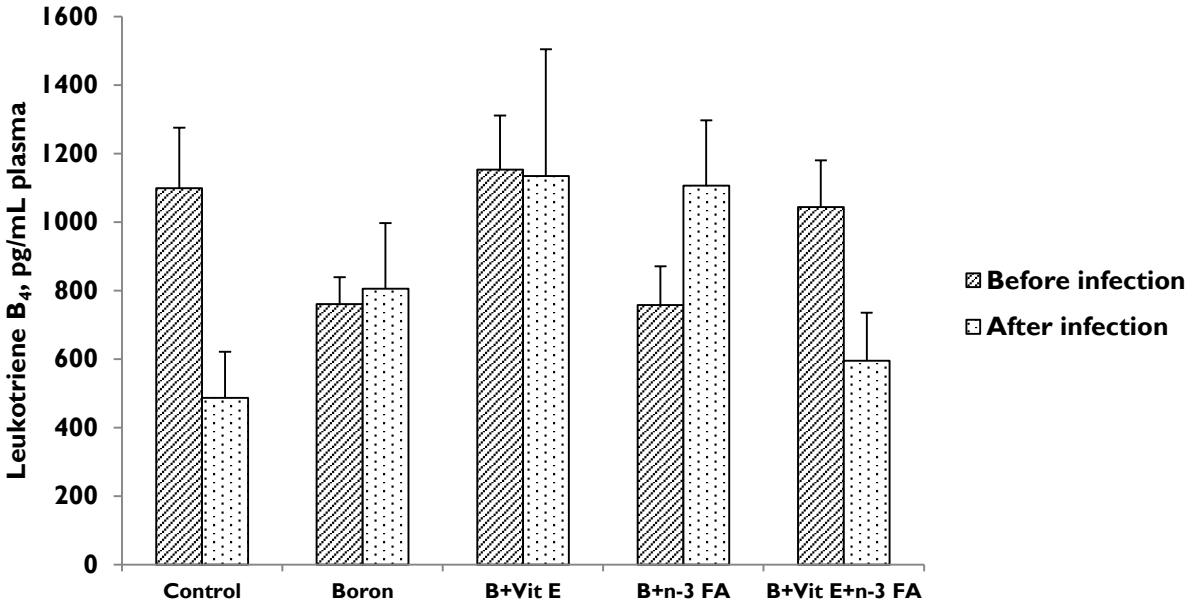

Figure 3. Infection by dietary treatment interaction for plasma concentration of leukotriene B4. The main effect of infection (P=0.220) and diet (P=0.270) were not significant while an interaction between infection and dietary treatments showed a trend (P=0.063).

Table 6 - Effect of supplementation of boron alone or with omega-3 fatty acids and 200 IU vitamin E on haematology measured before (day 6) and after (day 10) infection with an enterotoxigenic strain of E. coli.

	Infection		Diet						*P*=		
	Before	**After**	**Control**	**Boron**	**B+Vit E**	**B+n3FA**	**B+Vit E+n3FA**	**SEM**	**Infection**	**Diet**	**I x D**
Erythrocytes, $x10^{12}$/L	6.68	6.61	6.59	6.56	6.71	6.67	6.69	0.262	0.564	0.987	0.829
Leukocytes, $x10^9$/L	10.67	14.25	13.54	11.79	12.20	11.91	12.85	1.309	0.001	0.798	0.818
Lymphocyte, $x10^9$/L	5.24	6.56	5.92	5.82	5.51	5.28	6.98	0.419	0.001	0.016	0.358
Neutrophil, $x10^9$/L	4.87	6.68	6.65	5.32	6.10	5.87	4.93	1.166	0.001	0.786	0.691
Neutrophil:Lymphocyte	0.948	1.098	1.179	0.946	1.140	1.111	0.740	0.2145	0.114	0.461	0.432
Monocyte, $x10^9$/L	0.38	0.81	0.73	0.44	0.41	0.59	0.81	0.159	0.001	0.08	0.325
Eosinophil, $x10^9$/L	0.16	0.18	0.22	0.19	0.17	0.16	0.13	0.055	0.195	0.804	0.558
Basophil, $x10^9$/L	0.012	0.019	0.016	0.019	0.018	0.012	0.012	0.007	0.035	0.873	0.267
Platelet, $x10^9$/L	471	530	538	512	490	445	518	37.4	0.003	0.315	0.158
Haemoglobin, g/dL	13.7	13.4	13.3	13.5	13.2	13.9	13.8	0.61	0.245	0.851	0.919

Table 7 - Effect of supplementation of boron alone or with omega-3 fatty acids and 200 IU vitamin E on plasma urea, haptoglobin and leukotriene B_4 content measured before (day 6) and after (day 10) infection with an enterotoxigenic strain of E. coli.

	Infection		Diet						*P*=		
	Before	**After**	**Control**	**Boron**	**B+Vit E**	**B+n3FA**	**B+Vit E+n3FA**	**SEM**	**Infection**	**Diet**	**I x D**
Plasma urea, mmol/L	2.59	3.53	3.27	2.89	2.76	2.56	3.83	0.454	0.003	0.112	0.617
Haptoglobin, mg/mL	0.83	0.82	0.96	0.67	0.85	0.78	0.96	0.145	0.916	0.898	0.760

5.6. Intestinal histology

Dietary treatment did not alter the epithelial integrity in any part of the small and large intestine, although there was an approaching trend that pigs fed the B+*n*-3 fatty acids diet tended to increase crypt depth in the ileum compared with pigs fed a control diet (P=0.105, Table 8).

Table 8 - Effect of supplementation of boron alone or with n-3 fatty acids and 200 IU vitamin E on histology of the small and large intestine.

	Control	Boron	B+Vit E	B+n3FA	B+Vit E+n3FA	SEM	*P*=
Duodenum							
Villous height, μm	461	464	455	444	397	23.6	0.270
Crypt depth, μm	118	118	123	116	118	7.4	0.978
VH:CD, ratio	3.96	4.00	3.75	3.91	3.42	0.267	0.536
Muscular thickness, μm	391	405	393	356	340	38.6	0.730
Jejunum							
Villous height, μm	372	365	356	391	320	27.2	0.464
Crypt depth, μm	147	130	139	143	129	12.3	0.797
VH:CD, ratio	2.70	2.86	2.95	2.83	2.57	0.346	0.944
Muscular thickness, μm	311	285	311	232	279	26.2	0.227
Ileum							
Villous height, μm	358	371	334	372	334	21.3	0.538
Crypt depth, μm	93	127	114	136	105	11.6	0.105
VH:CD, ratio	4.15	3.16	2.96	3.03	3.33	0.436	0.331
Muscular thickness, μm	339	407	349	410	382	37.0	0.554
Colon							
Crypt depth, μm	233	248	231	227	235	19.4	0.951
Muscular thickness, μm	455	492	426	511	468	47.6	0.752

5.7. Relative mRNA expression of the tight junction protein and cyclooxygenase-2

Dietary treatment did not affect mRNA expression of tight junction proteins and COX-2 in the ileum (P<0.05). However, pigs fed the B+Vit E+*n*-3 fatty acids diet tended to increase occludin mRNA expression (P=0.105), increased ZO-1 mRNA expression (P<0.05), and tended to increase COX-2 expression (P=0.094, Table 9) in the colonic epithelium.

5.8. Diamine oxidase (DAO), malondialdehyde (MDA), prostaglandin E_2 (PGE_2) and total glutathione (tGSH) contents in the lever and ileal epithelium

Pigs fed the B+Vit E+*n*-3 fatty acids diet significantly increased their DAO content in the liver compared with pigs fed the boron or B+*n*-3 fatty acids diets (P<0.05). Pigs fed the B+Vit E and B+*n*-3 fatty acids diets tended to increase tGSH content in the liver compared with pigs fed the boron diet (P=0.083, Table 10). Pigs fed the B+Vit E+*n*-3 fatty acids diet tended to increase PGE_2 content in the ileal epithelium compared with pigs fed the control, B+Vit E, or B+*n*-3 fatty acids diets (P=0.066). Dietary treatment had no significant effect on MDA content in the liver and ileal epithelium.

Table 9 - Relative expressions (Pfaffl ratio) of the tight junction proteins and cyclooxygenase-2 mRNA in the ileal and colonic epithelium of pigs fed diets supplemented with boron alone or with omega-3 fatty acids and 200 IU vitamin E and experimentally infected with an enterotoxigenic strain of E. coli.

	Control	Boron	B+Vit E	B+n3 FA	B+Vit E+n3FA	SEM	*P*=
Ileum							
Occludin	1.00	1.04	0.98	1.27	1.11	0.203	0.878
ZO-1	1.00	1.45	2.00	2.00	1.18	0.437	0.409
COX-2	1.00	2.42	4.24	4.65	1.31	1.382	0.290
Colon							
Occludin	1.00	1.10	0.92	1.09	1.69	0.204	0.105
ZO-1	1.00[a]	1.45[ab]	1.09[a]	1.25[a]	1.84[b]	0.196	0.049
COX-2	1.00	1.05	1.92	1.16	5.03	1.136	0.094

Table 10 - Concentration of diamine oxidase (DAO), malondialdehyde (MDA), prostaglandin E2 (PGE2), and total glutathione (tGSH) in the liver and ileal epithelium of pigs fed diets supplemented with boron alone or with omega-3 fatty acids and 200 IU vitamin E and experimentally infected with an enterotoxigenic strain of E. coli.

	Control	Boron	B+Vit E	B+n3FA	B+Vit E+n3FA	SEM	*P*=
Liver							
DAO, μg/g wet tissue	199[ab]	180[a]	218[ab]	180[a]	239[b]	15.0	0.044
MDA, μmol MDA equivalent	3.96	13.15	6.35	7.65	4.32	3.132	0.264
PGE_2, μg/g wet tissue	26	32	22	34	25	3.8	0.170
tGSH, μg/g wet tissue	3772[ab]	3335[a]	4138[b]	3949[b]	3609[ab]	201.6	0.083
Ileum							
DAO, ng/g wet tissue	735	745	771	833	735	39.6	0.431
MDA, μmol MDA equivalent	4.42	3.92	3.33	3.40	3.71	0.310	0.140
PGE_2, μg/g wet tissue	108[a]	126[ab]	71[a]	105[a]	200[b]	30.0	0.066
tGSH, μg/g wet tissue	4226	3812	4004	3704	4279	290.7	0.537

5.9. Correlation analyses between boron, vitamin E and n-3 fatty acids intake per day per metabolic body weight, and measured variables

Boron intake was positively correlated with whole body weight at day 10 after weaning (r=0.469, P<0.05), villous height in the jejunal epithelium (r=0.479, P<0.05) and villous height to crypt depth ratio (r=0.339, P<0.10), and was negatively correlated with DAO content in the liver (r=-0.440, P<0.05, Table 11). Vitamin E intake was positively correlated with total glutathione content in the ileal epithelium (r=0.366, P<0.10), mRNA expression of occludin (r=0.553, P<0.05), ZO-1 (r=0.430, P<0.05), and COX-2 (r=0.544, P<0.05). Intake of *n*-3 FA was positively correlated with whole body weight at day 10 after weaning (r=0.412, P<0.05), villous height in the jejunal epithelium (r=0.498, P<0.05), villous height to crypt depth ratio (r=0.330, P<0.10), PGE_2 content in the liver (r=0.334, P<0.10), and was negatively correlated with DAO content in the liver (r=-0.454, P<0.05).

Table 11 - Correlation coefficients between intakes of boron, vitamin E and n-3 FA and measured variables.

Variables[1]		**Boron intake, mg/d/BW$^{0.75}$**	**Vitamin E intake, IU/d/BW$^{0.75}$**	**n-3 FA intake, mg/d/BW$^{0.75}$**
Body weight	Whole body weight	0.469*	0.085	0.412*
	Empty body weight	0.368†	0.154	0.306
Jejunum	Villous height	0.479*	0.035	0.498**
	VH:CD ratio	0.339†	0.147	0.330†
Ileum	Occludin mRNA	0.176	0.017	0.250
	ZO-1 mRNA	0.360	0.108	0.369
	DAO, ng/g wet tissue	0.120	-0.091	0.230
	PGE_2, μg/g wet tissue	0.015	0.124	0.070
	COX-2 mRNA	0.70	-0.062	0.221
	MDA, μmols/g wet tissue	0.062	0.140	0.005
	tGSH, μg/g wet tissue	0.139	0.366†	0.078
Colon	Occludin mRNA	0.200	0.553**	0.295
	ZO-1 mRNA	0.222	0.430*	0.252
	COX-2 mRNA	0.086	0.544**	0.076
Liver	DAO, μg/g wet tissue	-0.440*	0.221	-0.454*
	PGE_2, μg/g wet tissue	0.270	-0.288	0.334†
	MDA, μmols/g wet tissue	0.122	-0.220	-0.067
	tGSH, μg/g wet tissue	0.147	0.238	0.094
Plasma	LTB_4, Day 6	-0.089	0.213	-0.167
	LTB_4, Day 10	0.148	0.005	0.143
	Haptoglobin, mg/mL, Day 6	0.018	-0.019	0.159
	Haptoglobin, mg/mL, Day 10	-0.190	-0.240	-0.090

[1]COX-2: cyclooxygenase-2, DAO: diamine oxidase, LTB_4: Leukotriene B_4, MDA: malondialdehyde, PGE_2: prostaglandin E_2, VH:CD ratio: villous height to crypt depth ratio

5.10. Experiment 2: performance responses of commercially-housed finisher pigs to individual or combined supplementation of dietary boron, vitamin E and n*-3 fatty acids*

Performance responses of commercially-housed finisher pigs to dietary supplementation of boron, vitamin E and n-3 fatty acids are presented in Table 12. Over the 48 days of feeding pigs fed a combination of B+Vit E, B+n-3 fatty acids, or B+Vit E+n-3 fatty acids consumed significantly less amount of feed (P<0.01) that resulted in an inferior growth rate compared with pigs fed either control or boron diets (P<0.001). Pigs fed B+n-3 fatty acids and B+Vit E+n-3 fatty acids had thinner backfat thickness compared with pigs fed the other treatments after the data were corrected for variable slaughter weight (P<0.001).

Table 12 - Performance of the commercially-housed finisher pigs fed experimental diets (Experiment 2).

Treatment	Control	Boron	B+Vit E	B+n-3 FA	B+Vit E+n-3FA	SEM	*P=*
Initial weight, kg	52.2	52.2	52.2	52.2	52.2	1.17	1.000
Day 32 weight, kg	85.6	85.8	83.3	82.1	84.2	1.45	0.327
Day 48 weight, kg	107.6	106.2	103.2	102.5	103.9	1.54	0.126
Day 0-32							
ADG, kg	1.04^{a}	1.05^{a}	0.97^{bc}	0.93^{c}	1.00^{ab}	0.023	0.003
VFI, kg	2.23^{a}	2.26^{a}	2.10^{b}	2.05^{b}	2.15^{ab}	0.043	0.007
FCR, kg/kg	2.14	2.15	2.16	2.20	2.15	0.035	0.721
Day 33-48							
ADG, kg	1.32	1.27	1.25	1.28	1.24	0.036	0.071
VFI, kg	3.46^{a}	3.40^{ab}	3.23^{bc}	3.19^{c}	3.27^{bc}	0.066	0.024
FCR, kg/kg	2.52	2.68	2.60	2.50	2.68	0.061	0.123
Day 0-48							
ADG, kg	1.15^{a}	1.13^{a}	1.06^{b}	1.05^{b}	1.08^{b}	0.016	0.001
VFI, kg	2.62^{ab}	2.63^{a}	2.47^{c}	2.41^{c}	2.49^{bc}	0.045	0.003
FCR, kg/kg	2.27	2.34	2.32	2.30	2.31	0.031	0.628
No. of deaths	1.27	1.36	1.55	1.09	0.27	0.387	0.180
Off trial	0.00	0.09	0.00	0.09	0.09	0.074	0.736
HSCW, kg[1]	82.6	79.8	80.0	77.6	78.1	1.31	0.091
Dressing, %[1]	76.3	75.3	76.5	75.9	76.2	0.33	0.065
P2 Backfat, mm[1]	12.9^{a}	13.0^{a}	12.8^{a}	11.9^{b}	11.5^{b}	0.25	0.001
Loin depth, mm[1]	53.5	52.4	53.5	53.2	53.7	0.75	0.759

[1]Day 48 weight was used as a covariate for statistical analysis.

6. Discussion

Experimental infection with ETEC loosened faecal consistency, plasma urea content and numbers of leukocytes, lymphocytes, neutrophils, monocytes, basophils and platelets. However, infection with ETEC did not increase plasma haptoglobin content measured at 24 hours after infection, which is unusual compared with the previous studies conducted at Medina Research Station using the same strain of ETEC. Therefore, the pig's immune response to the ETEC infection was somewhat different from that of other previous experiments. It is unknown whether changes of immunity or passive immunity of the piglets at weaning played a role in this discrepancy. However, given both the loosened faecal score and the numbers of leukocytes, it is evident that the infection model elicited diarrhoea but might not have been severe enough to elicit overly strong immune system activation.

The first hypothesis tested was that dietary boron would improve intestinal barrier function through down-regulation of eicosanoid-mediators, as a number of publications reported that boron (1) inhibits PGE_2 and LTB_4 syntheses in rat leukocytes (Rajendran et al., 1994; Hunt and Idso, 1999; Nielson *et al*., 2007), (2) reduces ROS produced by neutrophils during immune stress (Hunt and Idso, 1999), and (3) inhibits serine proteases that produced by granulocytes and cause tissue damage during immune response (Hunt, 2003). Pigs fed diets with included boron at 7.5 ppm had higher boron contents in plasma and in the liver, indicating boron intake was consistent among the treatment groups except the control group. It seems boron supplementation alone or in combination with Vit E or *n*3- fatty acids decreased the numbers of monocytes compared with pigs fed a control diet or the diet supplemented with boron, vitamin E and *n*3- fatty acids. Apart from that, boron supplementation had no effect on the synthesis of eicosanoid mediators, biomarkers of antioxidant capacity (MDA, tGSH), intestinal structure, and tight junction protein expression in the ileum and colon. Therefore, the hypothesis that dietary boron will improve intestinal barrier function through down-regulation of eicosanoid-mediators was not supported in this experiment.

Interestingly, pigs fed the diet supplemented with all of the supplements (boron, vitamin E and n3- fatty acids) significantly increased their numbers of lymphocytes and the content of diamine oxidase (DAO) in the liver, increased PGE_2 synthesis in the ileal epithelium, and tended to have a higher COX-2 mRNA expression in the colon and increased ZO-1 mRNA expression in the colon compared with the pigs fed a control diet. Diamine oxidase is abundant in the intestinal mucosa and oxidative deamination of the essential polyamines that are used for cell proliferation is the major function (Wolvekamp and de Bruin, 1994). It is known to increase during adaptive hyperplasia found in small intestine resection or ulcerative colitis and hence considered as a biomarker for intestinal damage (Mennigen *et al*., 1990; Fukudome *et al*., 2014). In a small intestine resection study in rat, it was reported that administration of a DAO inhibitor, aminoguanidine, enhanced intestinal proliferation measured as intestinal weight and mucosal DNA content (Rokkas *et al*., 1990). Therefore, increased numbers of lymphocytes, DAO content in the liver, PGE_2 synthesis in the ileal epithelium, and COX-2 mRNA expression in the colonic epithelium of the pigs fed the B+Vit E+n-3 fatty acids diet indicate that combined supplementation of those additives increased the inflammatory response compared with the pigs fed the other diets. Based on this finding, increased ZO-1 mRNA expression in the colonic epithelium in pigs fed the B+Vit E+n-3 fatty acids diet is perhaps questionable. It is not clear whether the ZO-1 mRNA expression in the pigs fed the B+Vit E+n-3 fatty acids diet was stimulated by increased inflammatory response or due to increased repair of the epithelium after an inflammatory response. Therefore, further investigation is required to examine suitability of tight junction protein gene expression as a biomarker for intestinal barrier function.

The second hypothesis tested was that supplementation of vitamin E and omega-3 fatty acid with boron will synergistically down-regulate eicosanoid mediators. This hypothesis was based on reports that dietary boron and *n*-3 fatty acids additively reduced $PGF_{2}\alpha$ synthesis in cell damage caused by the ROS in rats (Nielsen et al., 2007; Nielsen 2009). Omega-3 fatty acids are well known to reduce production of eicosanoid mediators due to competitive conversion of linoleic acids (*n*-6 fatty acid) to arachidonic acids which are used as a precursor for production of eicosanoid mediators. Another beneficial effect of feeding *n*-3 fatty acids is that the membrane phospholipid embedded-*n*-3 fatty acids (eicosapentaenoic acids) are used for production of anti-inflammatory prostaglandin families such as PGE_3 when pathogen/antigen invasions are detected, while membrane phospholipid embedded *n*-6 fatty acids (arachidonic acids) are converted to immunosuppressive PGE_2 and LTB_4 (Goddeeris *et al*., 2002). As different mechanisms are involved in the anti-inflammatory effects of boron and other suggested nutrients such as *n*-3 fatty acids, and vitamin E, there is a need to understand the nature of synergism that might exist between boron, vitamin E, and *n*-3 fatty acids, which are all known to reduce PGE_2 production possibly through different mechanisms in pigs. However, results from this experiment showed that pigs fed B+Vit E and B+n-3 fatty acids only improved total glutathione content in the liver and reduced number of monocytes compared with pigs fed the control diet, and these improvements were not associated with intestinal barrier such as histology and epithelial DAO content and tight junction protein expression. Based on these results, the second hypothesis was also not supported.

As the study was designed to examine additive effects of the supplements, the effects of individual supplements on measured variables were difficult to observe. Therefore, intakes of boron, vitamin E and *n*-3 fatty acids by individual pig's per day were calculated based on the feed intake and analysed contents of boron, vitamin E and *n*-3 FA in the diets. Then the values were expressed as g intake per kg metabolic body weight-basis to eliminate the variations in individual feed intake due to differences in metabolically active body weight. These intake data were then analysed by correlation with the measured variables in an attempt to elucidate the impacts of the individual supplements on measured variables. The correlation study indicated that boron intake has significant positive effects on histology of the jejunum and whole and empty body weight, while it had a negative correlation with DAO content in the liver. These correlations suggest that intake of boron enhanced intestinal structure, reduced damage in the liver and increased body weight of pigs, although the effects were not detectable in the treatment-based statistical analysis. Vitamin E intake tended to positively correlate with total glutathione content in the ileal epithelium mainly due to antioxidant capacity of vitamin E. Vitamin E intake also had a positive correlation with tight junction protein mRNA expression in the colon. However, vitamin E intake also had a positive correlation with COX-2 mRNA expression in the colon, which was unexpected. This unexpected results can partly be explained by an *ex-vivo* study using the human Caco-2 cell line which demonstrated that α-tocopherol reduced COX-2 activity post-transcriptionally rather than affecting transcription of COX-2 mRNA (O'Leary *et al*., 2004). Omega-3 fatty acid intake was positively correlated with whole body weight at the end of the experiment and the jejunal villous height, and was negatively correlated with DAO content in the liver, indicating benefits of *n*-3 FA on preventing liver damage and better maintaining intestinal morphological structure.

In conclusion, although the correlation analysis showed a number of positive relationships between intake of boron, vitamin E and *n*-3 fatty acids on intestinal structure, tight junction protein expression and empty body weight, the experimental hypotheses were not supported. Therefore, it is concluded that dietary inclusion of boron, vitamin E and *n*-3 FA in diets for weaner and finisher pigs had limited impacts on intestinal health and performance. However, given the positive relationship between intake

of boron, vitamin E and *n*-3 fatty acids and some of the indicators for intestinal health, further refined research is warranted to elucidate whether concentrations of boron in the diet would have impact on intestinal health and performance of pigs.

7. Implications & Recommendations

Supplementation of boron, vitamin E and *n*-3 fatty acids in combination in diets for weaner and finisher pigs had limited impact on the indicators of intestinal health and performance of pigs. However, positive correlations between intake of boron and intestinal health need further investigation to elucidate whether inclusion level of boron in diets for weaner pigs would have positive impact on intestinal health and performance with and without infection stress.

8. Intellectual Property

There were no intellectual properties arising from this experiment.

9. Technical Summary

No additional technical information on methodologies, equipment design etc was developed as part of these experiments.

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11. Publications Arising

There are no publications arising at this point. An abstract was submitted to the 2015 Australasian Pig Science Association meeting.