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Reducing the dietary omega-6 to omega-3 polyunsaturated fatty acid ratio attenuated inflammatory indices and sustained epithelial tight junction integrity in weaner pigs housed in a poor sanitation condition

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Highlights

- A total of 108 male pigs [Duroc × (Yorkshire × Landrace); initial BW 7.1 ± 0.5 kg] weaned at 21 days of age were randomly allocated to one of 3 dietary treatments and 2 environmental conditions (sanitary vs. unsanitary) (6 replicate pens per treatment, 3 pigs per pen)
- One pig per pen (n = 6) was euthanized on d 0, d 7 and d 14, to collect blood and small intestinal tissue samples
- Housing pigs in an unsanitary environment after weaning increased inflammatory responses and reduced growth performance, but reducing the n-6:n-3 PUFA ratio to 4:1 attenuated the inflammatory responses induced by the unsanitary environment.

ABSTRACT

The present study was conducted to determine the effect of reducing dietary n-6:n-3 PUFA ratio on the performance, inflammatory response and gut morphology of PWD challenged with sanitary and poor sanitary conditions in weaned pigs, and to test the hypotheses that (1) exposure to an poor sanitary environment will increase indices for

inflammatory response; and (2) reducing n-6:n-3 PUFA ratio in diets for weaned pigs will attenuate the inflammatory response induced by the environmental challenge. A total of 108 male pigs [Duroc × (Yorkshire × Landrace); initial BW 7.1 ± 0.5 kg] weaned at 21 days of age were randomly allocated to one of 3 dietary treatments and 2 environmental conditions (sanitary vs. poor sanitary) to give 6 replicate pens per treatment with 3 pigs per pen. The dietary treatments were 3 graded levels of n-6:n-3 PUFA ratio (i.e.,20:1, 10:1 and 4:1) formulated using tallow, safflower oil, and a vegetable and fish oil blended product. One pig per pen (n = 6) was euthanized on d 0, d 7 and d 14, to collect blood and small intestinal tissue samples. Pigs exposed to a poor sanitary environment tended ($P<0.10$) to grow more slowly and utilized feed less efficiently ($P<0.05$) compared with the pigs housed in sanitary conditions. Housing weaned pigs in a poor sanitary environment increased ($P<0.05$) the incidence of diarrhoea. Furthermore, a poor sanitary environment increased ($P<0.001$) the occludin diffusion in the ileal epithelium of weaned pigs and increased plasma concentrations of TNF-α ($P<0.05$), COX-2 ($P<0.05$), PGE2 ($P<0.01$) and LTB4 ($P<0.05$) on d 14. Reducing the n-6:n-3 PUFA ratio improved ($P<0.05$) both ADG and FCR but reduced ($P<0.01$) the incidence of diarrhoea over 14 days after weaning, and they tended to attenuate ($P<0.10$) the diffusion of the transmembrane tight junction protein occludin at the apical intercellular region of the ileal epithelium. Moreover, reducing the n-6:n-3 ratio in the diet attenuated the increased inflammatory indices induced by the environmental challenge. Correlation analysis indicated that n-6 PUFA intake of individual pigs positively correlated with plasma concentrations of IL-1β ($P<0.01$), TNF-α ($P<0.05$), PGE_2 ($P<0.01$) and COX-2 ($P<0.05$). Our results indicated that housing pigs in a poor sanitary environment after weaning increased inflammatory responses and reduced growth performance. Reducing the n-6:n-3 PUFA ratio to 4:1 attenuated the inflammatory responses observed after weaning in both environment on d 7 and in the poor sanitary environment on d 14.

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ANOVA, analysis of variance; BW, body weight; COX-2, cyclooxygenase-2; CP, crude protein; D, dietary treatments; DE, digestible energy; DHA, docosahexaenoic acid; DM, dry matter; E, environmental condition (sanitary vs poor sanitary); ELISA, enzyme linked immunosorbent assay; EPA, eicosapentaenoic acid; FCR, feed conversion ratio; GE, gross energy; IL-1β, interleukin 1β; LTB_4, leukotriene B_4; N, nitrogen; n-6:n-3, omega-6 to omega-3; PGE_2, prostaglandin E_2; PPARγ, peroxisome proliferator-activated receptor-γ; PUFA, polyunsaturated fatty acid; PWD, post-weaning diarrhoea; SEM, standard error of mean; SID, standardised ileal digestible; TNF-α, tumor necrosis factor α; V:C, villous height: crypt depth.

Keywords: Diarrhoea; Growth performance; Inflammatory response; Omega 6 to 3 polyunsaturated fatty acid ratio; Pig; Weaning

1. Introduction

Weaner pigs are exposed to a number of stressors including microbial and (or) viral challenges. The association between stress and continuous exposure to pathogens can damage the intestinal epithelium, and lead to secondary inflammation and activation of the immune system (Lallès et al., 2007; Kim et al., 2012). Inflammation and subsequent immune system activation stimulates release of pro-inflammatory cytokines (Kim et al., 2013) that in turn stimulate the neurological lipid (eicosanoid) signaling system, which produces prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) through actions of cyclooxygenase and lipoxygenase. Production of PGE_2 and LTB_4 is known to increase metabolic rate and heat production and trigger anorexia (Rakhshandeh and de Lange., 2011).

Caughey et al. (1996) demonstrated an inverse exponential relationship between eicosapentaenoic acid (EPA; 20:2n-3) content in mononuclear cell and pro-inflammatory cytokine production in humans fed either flaxseed oil or fish oil for 4 weeks. This study reported decreased pro-inflammatory cytokine synthesis when cellular EPA concentration was increased to over 1% of total membrane fatty acids. When inflammation and pro-inflammatory cytokines stimulate membrane cell walls, α-linolenic acids (18:3n-3) are converted to EPA which produces anti-inflammatory prostaglandins (3-series prostaglandins) and leukotrienes (5-series leukotriene), while linoleic acids (18:2n-6) are converted to arachidonic acid (20:4n-6). Arachidonic acid, in turn, produces pro-inflammatory prostaglandins (2-series prostaglandins) and leukotrienes (4-seies leukotrienes) (Wall et al., 2010). Therefore, one of the suggested nutritional strategies both in humans (Simopoulos., 2008) and pigs (Wilkinson et al., 2011) to mitigate inflammatory responses is to reduce the omega 6 to omega 3 polyunsaturated fatty acid (PUFA) ratios in the diet. In a human study, a omega-6 to omega-3 (n-6:n-3) PUFA ratio less than 4:1 has been recommended to reduce the risks of chronic diseases (Simopoulos., 2008). Similarly, a recent finishing pig study demonstrated that dietary n-6:n-3 ratios of 1:1 or 5:1 efficiently suppressed circulating levels of inflammatory cytokines compared with pigs fed a diet with a 10:1 ratio (Duan et al., 2014). However, relationships between the dietary n-6:n-3 PUFA ratio and inflammatory responses in pigs under environmental challenges, such as increased pathogen exposure due to poor sanitary housing conditions, have not been reported. Therefore, the present study tested the hypotheses that (1) exposure to a poor sanitary environment will increase indices for inflammatory response; and (2) reducing the n-6:n-3 PUFA ratio in diets for weaned pigs will attenuate the inflammatory response induced by the environmental challenge.

2. Materials and methods

The experimental protocol used in this study was approved by the Animal Ethics Committee of the Chungnam National University (CNU-00485).

2.1. Experimental design, animals, housing and diets

The experiment was designed as a 2 × 3 factorial arrangement of treatments, with the respective factors being 1) two sanitary environments (Sanitary: cleaned and disinfected previously unpopulated rooms; poor sanitary: manure application to plastic-covered expanded metal flooring with no cleaning or disinfection of a previously populated room) to permit a contrasting environment regarding to inflammation; and 2) three ratios of n-6:n-3 PUFA (4:1, 10:1 and 20:1) which were decided based on the previous study reports (Duan et al., 2013; Eastwood et al., 2014) with little modifications.

A total of 108 male pigs [Duroc × (Yorkshire × Landrace)] weaned at 21 days (d) of age with initial body weight (BW) of 7.1 ± 0.5 kg (mean ± SEM) were used. Pigs were obtained from a local government experimental farm (Chungnam Livestock Research Institute, Cheongyang, Chungnam) at weaning and transported to an animal facility at Chungnam National University. Pigs were allocated to their experimental treatments based on initial BW and block within room in the animal facility. The facility contained two rooms that allowed pigs to be housed separately, i.e., in the sanitary and poor sanitary environments, to avoid any cross contamination. Six replicate pens of three pigs per pen were allocated to each treatment (3 pigs × 6 pens × 6 treatments = 108 pigs). Each pen was equipped with a nipple bowl drinker and a metal trough. The ambient temperature was maintained at 29 ± 1ºC for the initial week and then gradually decreased by 1ºC every week. Pigs were offered the experimental diets on an *ad libitum* basis for 2 weeks, and were freely accessible to the water at all times. Diets were formulated to meet or exceed NRC (2012) and it contain the same digestible energy (DE) content and crude fat contents but varying n-6:n-3 PUFA concentrations using tallow, safflower oil and a blend of vegetable and fish oil [REI 3®

(Morning Bio Ltd., Cheonan, South Korea) supplied per kilogram of total diets: n-3 PUFA 160 g (α-linolenic acid 147 g, docosahexaenoic acid; DHA 9 g and EPA 4 g)]. Diet composition and nutrient contents of the experimental diets are presented in Table 1. Feed intake was recoded on a weekly basis as feed disappearance from the feeder.

2.2. Measurement of BW and feed consumption, and sampling

The BW was measured individually at 0, 7, and 14 d of age. Feed consumption was recorded on a pen basis during the experiment to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

2.3. Assessment of fecal consistency and the incidence of diarrhoea

Feces were visually accessed daily at 10 am for 2 weeks after weaning to determine fecal consistency scores and the incidence of diarrhoea using the procedure described by Heo et al. (2009).

2.4 Post-mortem procedure

One pig per pen (n = 6) closest to the median BW was euthanized on day 0, day 7 and day 14 according to modified procedures described in Heo et al. (2010), to collect blood and intestinal tissue samples (1 pig per pen; a total of 18 pigs per each time period). Pigs were administered a single intramuscular injection of 0.1 mL SUCCIPHARM® (50 mg suxamethonium chloride, Komipharm International Co., Ltd., Shieung, South Korea) to induce general anesthesia, and then blood samples (5-10 mL) were collected via the jugular vein puncture into vacutainer tubes coated with lithium heparin. The blood plasma was separated by centrifugation at 2,000 × *g* for 10 min at 4°C and stored at -80°C until required. Thereafter, pigs were introduced into a chamber where residual air was rapidly flushed with 100% CO_2 until death was confirmed. The abdominal cavity was exposed by midline laparotomy. For measurement of villous height and crypt depth, a 3-4 cm section segment of

the small intestine was removed at the ileum (approximately 5 cm cranial to the ileo-caecal junction) as described by Heo et al. (2010) and carefully washed with phosphate buffered saline and preserved in 10% formalin solution for subsequent histological examination and immunofluorescence.

2.5. Mucosal histology

Tissue preparation and methods for villous height and crypt depth were conducted following standard histological procedures described by Heo et al. (2010). After fixation in 10% phosphate buffered saline for a few days, ring shaped lengths of ileum were excised carefully. Thereafter, dehydrated tissue samples were followed by embedding in paraffin wax. From each of these, 6 transverse sections (4-6 μm) were cut, stained with hematoxylin and eosin, and mounted on glass slides. The height of 10 well oriented villi and their associated crypts were measured with a light microscope (Eclipse TE2000, Nikon Instruments Inc., Melville, NY 11747-3064, USA) using a calibrated eyepiece graticule (Pluske et al., 1996; Heo et al., 2010).

2.6. Enzyme linked immunosorbent assay (ELISA) measurements

The concentrations of interleukin 1β (IL-1β; R&D Systems, Minneapolis, MN), tumor necrosis factor α (TNF-α; R&D Systems, Minneapolis, MN), PGE_2 (MyBioSource, San Diego, CA), LTB_4 (MyBioSource, San Diego, CA), and cyclooxygenase-2 (COX-2; MyBioSource, San Diego, CA) in plasma were quantified using commercially available ELISA kits according to the manufacturers' instructions described by Piñeiro et al. (2009).

2.7. Immunofluorescence

Ileum tissues were fixed in 4% paraformaldehyde, washed and embedded in paraffin. Tissue sections were cut, placed on slides, deparaffinized, rehydrated, blocked, and incubated with rabbit polyclonal anti-occludin antibody (ThermoScientific) and mouse monoclonal anti-actin antibody (Abcam) overnight at 4^{o}C, then washed, and incubated with

Alexa Fluor® 488 conjugated goat anti-rabbit IgG secondary antibody (ThermoScientific), TRITC conjugated goat anti-mouse IgG secondary antibody, and 4',6-diamidino-2-phenylindole (DAPI; DNA staining, Molecular Probes, Eugene, OR) for 40 min at room temperature. The slides were observed under fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Seoul, South Korea).

To determine whether sanitary challenge and (or) dietary n-6:n-3 PUFA ratio influenced inter-epithelial tight junction integrity, the localization of the transmembrane tight junction protein occludin was examined. Occludin localization was assessed using a three-scale visual assessment as described by Heo et al. (2009; 2010) with little modifications: Score 1 = occludin was localized to the apical intercellular region of ileal epithelium; Score 2 = occludin was partly localized to the apical intercellular region while some occludin was diffused at the intercellular tight junction region; Score 3 = Most of occludin was diffused at the intercellular tight junction region. Collected data were then expressed as the proportion of occludin diffused at the ileal epithelium. A higher diffusion score indicates more disruption of the inter-epithelial tight junction.

2.8. Chemical Analyses

Diet dry matter (DM) was determined according to the AOAC (method 925.09; AOAC, 1990) by oven drying 5 g of samples at 105°C overnight. The gross energy (GE) content of experimental diets was measured using an adiabatic bomb calorimeter (model 6300, Parr Instrument, Moline, IL, USA) that was calibrated using benzoic acid as a standard. The nitrogen (N) content in the experimental diets was determined by the combustion technique (method 990.03; AOAC, 1990) using the LECO N analyser (model CNS-2000; LECO Corp., St. Joseph, MI, USA), and crude protein (CP) was calculated as N × 6.25. Fatty acid concentrations were determined by gas chromatography with a flame ionization detector (Shimadzu Model 14A, Columbia, MD) (Park and Goins, 1994). Peaks were identified by

comparison of retention times of known standards including EPA and DHA. Quantification was corrected for recovery of the internal standard and is based on the reference standard.

2.9. Statistical analyses

Data were analysed as completely randomized block design, using the general linear model (GLM) procedure of analysis of variance (ANOVA; SPSS, Version 21, SPSS Inc., Chicago, Illinois, USA). The pig was the experimental unit for all measures except growth performance in which a pen was the experimental unit, and the model included dietary treatments and sanitary conditions as two main effects. Pigs were allocated based on weaning weight and the block was used as a random factor in the model for all measured experimental variables. The BW, ileum morphology and immune response on d 0 were included in the model as a covariate for analyses of growth performance data, ileum architecture and cytokine analyses, respectively. Data for the incidence of diarrhoea were expressed as the mean percentage of days with diarrhoea relative to the 14 days after weaning (Heo et al., 2009). Statistical significance was accepted at $P<0.05$. Tukey's multiple range test was used to compare between-treatment differences when there was a significant treatment effect.

3. Results

3.1. Growth performance

Pigs exposed to a poor sanitary environment tended ($P<0.10$) to grow more slowly and utilized the feed less efficiently ($P<0.05$) compared with the pigs housed in a sanitary environment. Reducing the n-6:n-3 PUFA ratio improved ($P<0.05$) ADG and FCR ($P<0.05$) for 14 days after weaning. Nonetheless, there was no interaction ($P>0.05$) between environmental conditions (sanitary vs poor sanitary) and dietary treatments (Table 2).

3.2. Ileal morphology and the incidence of diarrhoea

Sanitary conditions and dietary treatments had no effect ($P>0.05$) on morphology of the ileal epithelium on d 7 (Table 3). However, pigs housed in a poor sanitary condition

showed a decreased villous height to crypt depth ratio in the ileum ($P<0.05$) due to increased villous atrophy ($P<0.05$) and crypt hyperplasia ($P<0.05$), while dietary treatment had no effect on ileal morphology on d 14 after weaning. Housing weaned pigs in a poor sanitary environment increased ($P<0.05$) the incidence of diarrhoea, however, feeding diets with a reduced n-6:n-3 PUFA ratio reduced ($P<0.01$) the incidence of diarrhoea over 14 days after weaning (Table 4).

3.3. Mucosal immunohistology and occludin localization

The representative image of the tight junction protein occludin stained using immunofluorescence in the ileal epithelium is presented in Figure 1. The occludin localization using a three-scale assessment, which is expressed as the proportion of occludin diffused at the ileal epithelium, is presented in Table 4 (i.e., a higher diffusion score indicated more disruption of the inter-epithelial tight junction). Results showed pigs housed in a poor sanitary environment had a higher ($P<0.001$) occludin diffusion score, and reducing the dietary n-6:n-3 ratio to 4:1 tended ($P<0.10$) to decrease occludin diffusion score at the intercellular tight junction region.

3.4. Circulating cytokines and lipid mediators

On d 7, pigs housed in a poor sanitary environment tended ($P<0.1$) to show an increase in circulating IL-1β, and showed increased COX-2 ($P<0.05$), PGE_2 ($P<0.001$) and LTB_4 ($P<0.05$) levels. Reducing the n-6:n-3 PUFA ratio in the diet decreased ($P<0.01$) circulating PGE_2. On day 14, pigs housed in a poor sanitary room had higher circulating TNF-α ($P<0.05$), COX-2 ($P<0.05$), and LTB_4 ($P<0.01$). Reducing the n-6:n-3 PUFA ratio in the diet tended to decrease TNF-α ($P<0.10$) and COX-2 ($P<0.10$) concentrations, and decreased circulating LTB_4 ($P<0.01$) levels (Table 5).

There were interactions between sanitary challenge and the n-6:n-3 PUFA ratio on d 14 for IL-1β ($P<0.01$) and PGE_2 ($P<0.05$) such that increasing the n-6:n-3 ratio to 20:1

increased and PGE_2 ($P<0.01$) contents under poor sanitary environmental challenge compared with pigs in sanitary conditions, and however IL-1β ($P<0.05$) increased with higher the n-6:n-3 PUFA ratio independent of environmental conditions (Figure 2).

4. Discussion

The present study tested the hypothesis that reducing the n-6:n-3 PUFA ratio in diets for weaned pigs will attenuate the inflammatory response induced by an environmental challenge. This hypothesis was based on the assumption that exposure to a poor sanitary environment at weaning transition, along with depleted passive immunity as a consequence of the diet from sows' milk to the weaning diet, will increase secondary inflammation and the associated with immune response, and might lead pigs to the occurrence of diarrhoea in the population. Our data supported the hypothesis as housing pigs in a poor sanitary environment significantly increased villous atrophy, crypt hyperplasia, localization of tight junction protein at the ileal epithelium, the incidence of diarrhoea and inflammation indices such as IL-1β, TNF-α, PGE_2, COX-2, and LTB_4 compared with pigs housed in a sanitary environment. Consequently, pigs housed in a poor sanitary environment tended to grow more slowly (5.6%) and used feed less efficiently (4.5%) during the 14 days after weaning compared to pigs housed in a sanitary environment. Similar results were reported by Montagne et al. (2012) and Pastorelli et al. (2012), who both reported increased diarrhoea and 9% and 7% reductions in feed efficiency, respectively, when weaned pigs were housed in a poor sanitary environment after weaning. Montagne et al. (2012) also reported a negative impact of housing pigs in a poor sanitary environment on fecal microbial composition, with a poor sanitary environment causing a reduction in *Lactobacillus* numbers and the *Lactobacillus*:*Enterococcus* ratio. Collectively these studies, in conjunction with the present study, indicate that housing pigs in a poor sanitary environment precipitated a range of

physiological effects including epithelial damage, inflammatory responses and a higher incidence of diarrhoea compared with pigs housed in a sanitary environment.

Another assumption imposed using this experimental model was that the sanitary challenge will trigger the lipid signalling system and eicosanoid mediators will be elevated. Release of pro-inflammatory cytokines stimulates cell membranes where phospholipase C and A_2 produce 20-carbon n-6 PUFA arachidonic acids from diacylglycerol or phospholipids. Arachidonic acid is then converted to PGE_2 and LTB_4 via facilitation of COX-2 and lipoxygenase, respectively (Rivest., 2010; Kalinski., 2012). PGE_2 is known to provoke anorexia and fever through stimulation of the neuroendocrine system (Rivest, 2010), while LTB_4 controls the severity and duration of the inflammatory response (Devchand et al., 1996). The sanitary challenge applied in the present study was sufficient to trigger this lipid signaling system and significantly increased circulating levels of PGE_2 and LTB_4.

We further tested the assumption that the sanitary challenge-induced inflammatory responses could be attenuated by altering the dietary n-6:n-3 PUFA ratio, that in turn alters the production of anti-inflammatory prostaglandins or pro-inflammatory prostaglandins. The conversion of α-linolenic acids and linoleic acids to EPA and arachidonic acids results in competition for metabolism by the same enzymes Δ6 desaturase, elongase and Δ5 desaturase (Rossi et al., 2010). Therefore, reducing the n-6:n-3 PUFA ratio in the diet would reduce inflammatory responses in a competitive manner compared with diets containing higher ratios of n-6:n-3 PUFA ratio. Previous human studies proposed a n-6:n-3 ratio of 4:1 to improve metabolic diseases including cardiovascular disease (Simopoulos, 2002; 2006). In pigs, Wilkinson et al. (2011) fed diets containing either tallow-based saturated fatty acids, fish-oil-based omega-3 PUFA, or safflower-oil-based n-6 PUFA to gilt progeny. This particular study clearly demonstrated the importance of fatty acids on the health and well-

being of weaned pigs in a commercial production system, particularly in gilt progeny where passive immunity is inferior compared with sow progeny.

Subsequently, Duan et al. (2014) fed experimental diets containing either n-6:n-3 PUFA ratios of 1:1, 2.5:1, 5:1 and 10:1 to finishing pigs (73.8 kg initial weight). Daily gain and feed utilization efficiency were significantly greater in pigs fed the n-6:n-3 ratio of 5:1 compared with the other treatments. Increasing the n-6:n-3 PUFA ratio from 1:1 to 10:1 linearly increased serum concentrations of the pro-inflammatory cytokines IL-1β and IL-6. Moreover, gene expressions of the peroxisome proliferator-activated receptor-γ (PPARγ) ligands in both the *longissimus lumborum* muscle and subcutaneous adipose tissue were significantly higher in pigs fed the diets containing a n-6:n-3 PUFA ratio greater than 5:1 compared with pigs fed the diet containing n-6:n-3 PUFA ratio smaller than 2.5:1. The PPARγ ligands are known to inhibit production of IL-6, TNF-α and IL-1β (Jiang et al., 1998). In our study, a pig's n-6 PUFA daily intake was positively correlated to plasma concentrations of pro-inflammatory cytokines and eicosanoid mediators. This finding not only reinforces the notion that pigs subjected to greater bacterial and viral loads respond accordingly, but that limiting n-6 PUFA intake through diet formulation with saturated fatty acids and n-3 PUFA is desirable under such conditions.

In this regard, the present study clearly demonstrated that reducing the n-6:n-3 PUFA ratio in the diet for weaned pigs reduced plasma concentrations of pro-inflammatory cytokines and eicosanoid mediators on day 14 after weaning. Interaction between the n-6:n-3 PUFA ratio and the sanitary challenge were expected for circulating pro-inflammatory cytokines and eicosanoid mediators, as pigs in the sanitary environment would not trigger inflammatory responses unlike pigs in the poor sanitary environment. However, only IL-1β and PGE_2 showed significant interactions between sanitary challenge and the dietary ratio of n-6:n-3 PUFA on day 14. We anticipated significantly lower cytokine and eicosanoid levels

(i.e., IL-1β and PGE_2, respectively) according to a reduced dietary n-6:n-3 PUFA ratio where pigs encounter a bacterial challenge (i.e., poor sanitary environment). However, we found significantly higher levels (i.e., IL-1β and PGE_2) in pigs fed a diet with reduced dietary n-6:n-3 PUFA ratio in the poor sanitary environment compared to those in the sanitary environment (Figure 2). This unexpected result could partly be explained by stress responses of newly weaned pigs, which can induce secondary inflammation and subsequent immune responses (Stokes et al., 2004; McLamb et al., 2013; Kim et al., 2013).

Maintaining intestinal integrity and barrier function around weaning is important for optimum digestion and absorption of nutrients and reduction of inflammation caused from enteric pathogens. Weaning stressors and exposure to pathogenic microbial loads generally increases villous atrophy and crypt hyperplasia (Pluske et al., 1997). Such changes are known to reduce digestive and absorptive capacities and contribute to post-weaning diarrhoea. Zhao et al. (2007) observed decreased epithelial integrity in weaned pigs housed in a poor sanitary environment compared with pigs housed in a sanitary environment. Feeding diets containing plasma protein tended to improve the damaged intestinal integrity induced by poor sanitary environmental challenge (Zhao et al., 2007). Similarly, the present study demonstrated that a poor sanitary environmental challenge reduced the villous height to crypt depth ratio on d 14 after weaning, however the dietary n-6:n-3 PUFA ratio did not statistically improve the morphology of the ileum housed in a poor sanitary environment ($P>0.1$). Similar to Zhao et al. (2007), transmembrane tight junction protein occludin expression demonstrated only a trend for an improvement as a result of reducing the n-6:n-3 PUFA ratio in diets, while poor sanitary challenge markedly increased the diffusion of the tight junction protein occludin. However, de Vasconcelos Generoso et al. (2015) demonstrated a significant improvement in villous height to crypt depth ratio by supplementation of n-3 PUFA in the ileum of rats experimentally induced with mucositis. Conjointly, the intestinal damage induced by sanitary

challenge in the present study may have not been severe enough to demonstrate the differences by altering the dietary n-6:n-3 ratio, while clinical inflammation such as mucositis or *E. coli* inflammation in the pigs may respond better with dietary n-6:n-3 ratio.

5. Conclusion

The results of this study indicate that housing pigs in a poor sanitary environment significantly increased inflammatory responses and reduced growth performance, but reducing the dietary n-6:n-3 PUFA ratio to 4:1 attenuated the inflammatory responses (i.e., IL-1β and PGE_2) induced by the poor sanitary environment.

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Table 1 Composition of the experimental diets (g/kg, as-fed basis)

Item	Dietary treatment (n-6:n-3 ratios)		
Ingredients, g/kg	20:1	10:1	4:1
Barley	50.0	50.0	50.0
Maize	308.6	306.2	305.2
Wheat	100.0	100.0	100.0
Rolled oats	100.0	100.0	100.0
Soybean meal	150.0	150.0	150.0
Full fat soya	50.0	50.0	50.0
Blood meal	20.0	20.0	20.0
Fishmeal	67.1	67.3	67.4
Whey powder	100.0	100.0	100.0
Tallow	14.8	17.0	17.9
Safflower oil	17.0	4.8	0.0
Fish oil[1]	0.0	12.2	17.0
Lysine-HCl	3.1	3.1	3.1
DL-Methionine	2.3	2.3	2.3
L-Threonine	1.3	1.3	1.3
L-Tryptophan	0.7	0.7	0.7
Vitamin-mineral premix[2]	1.0	1.0	1.0
Limestone	4.1	4.1	4.1
Dicalcium phosphors	7.7	7.6	7.6
Salt	2.0	2.0	2.0
Choline Chloride	0.4	0.4	0.4
Calculated analysis			
CP, g/kg	222.0	222.0	221.0
DE, MJ/kg	15.5	15.5	15.5
SID Lysine, g/MJ DE	0.9	0.9	0.9
Crude fat, g/kg	76.2	76.2	76.2
Analysed chemical composition			
CP, g/kg	218.8	229.2	215.1
DM, g/kg	887	897	901
GE, MJ/kg	16.9	17.1	17.2
Total n-6[3], g/kg	54.8	53.8	44.5
Total n-3[4], g/kg	2.8	5.1	11.3
n-6:n-3 ratio	19.4:1	10.6:1	3.9:1

[1]REI 3® (Morning Bio Ltd., Cheonan, South Korea) supplied per kilogram of total diets: omega 3 fatty acid 160 g (α-linolenic acid 147 g, docosahexaenoic acid 9 g and eicosapentaenoic acid 4 g)

[2]Provided the following nutrients (per kg of air-dry diet):

Vitamins: A 7000 IU, D_3 1400 IU, E 20 mg, K 1 mg, B_1 1 mg, B_2 3 mg, B_6 1.5 mg, B_{12} 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 sulphate), Cu 10 mg (as copper sulphate), iodine 0.5 mg (as potassium iodine), iron 60 mg (as ferrous sulphate), Mn 40 mg (as manganous oxide), Se 0.3 mg (as sodium selenite), Zn 100 mg (as zinc oxide). (BJ Grower 1, BioJohn Pty Ltd., WA, Australia)

[3]Total n-6; C18:2+ C18:3+ C20:4

[4]Total n-3; C18:3n3+ C18:4+ C20:5+ C22:5+ C22:6

Table 2. Effects of sanitary challenge and dietary n-6:n-3 PUFA ratio on growth performance in weaned pigs[1]

Item	Environmental conditions		SEM[2]	n-6:n-3 ratio			SEM	P-value[3]		
	Sanitary	poor sanitation		20:1	10:1	4:1		E[4]	D[5]	E × D[6]
Day 1-7										
ADG, g	91	87	13.6	76	92	100	2.7	†	*	NS
ADFI, g	174	174	18.7	162	178	182	4.9	NS	NS	NS
FCR, g/g	1.93	2.00	0.29	2.14	1.95	1.82	0.08	*	*	NS
Day 8-14										
ADG, g	263	247	27.8	241	257	267	4.5	*	*	NS
ADFI, g	374	376	16.3	362	378	387	3.7	NS	NS	NS
FCR, g/g	1.42	1.52	0.15	1.45	1.46	1.42	0.03	*	†	NS
Day 1-14										
ADG, g	177	167	19.3	158	174	183	2.8	†	*	NS
ADFI, g	274	275	12.4	261	278	284	2.3	NS	NS	NS
FCR, g/g	1.68	1.76	0.18	1.82	1.71	1.63	0.04	*	*	NS

[1]Values for day 1-7 and d 8-14 are the mean of 6 replicates with 36 and 18 pigs, respectively

[2]Pooled standard error of mean

[3]Significance level: NS: Not significant, † P<0.1, * P<0.05, ** P<0.01, *** P<0.001

[4]Environmental condition (sanitary vs poor sanitary)

[5]Dietary treatment

[6]Interaction between dietary treatment and environmental condition

Table 3. Effects of sanitary challenge and dietary n-6:n-3 PUFA ratio on ileal morphology and diarrhoea index in weaned pigs

Item	Environmental conditions		SEM[1]	n-6:n-3 ratio			SEM	P-value[2]		
	Sanitary	poor sanitary		20:1	10:1	4:1		E[3]	D[4]	E × D[5]
Day 7										
Villous height (µm)	427	423	14.8	423	422	430	10.5	NS	NS	NS
Crypt depth (µm)	198	208	9.8	204	202	203	7.9	NS	NS	NS
V:C[6]	2.2	2.0	0.01	2.1	2.1	2.1	0.01	NS	NS	NS
Day 14										
Villous height (µm)	572	425	11.6	504	499	492	14.2	*	NS	NS
Crypt depth (µm)	253	212	13.2	230	226	241	15.2	*	NS	NS
V:C[6]	2.3	2.0	0.01	2.2	2.2	2.0	0.01	*	NS	NS
Diarrhoea index[7], %	7.3	27.7	0.31	25.3	19.3	8.0	0.25	*	**	NS

[1]Pooled standard error of mean

[2]Significance level: NS: Not significant, † P<0.1, * P<0.05, ** P<0.01, *** P<0.001

[3]Environmental condition (sanitary vs poor sanitary)

[4]Dietary treatment

[5]Interaction between dietary treatment and environmental condition

[6]V:C, villous height: crypt depth

[7]The diarrhoea index was expressed as a proportion of days with diarrhoea with respect to total number of days (14 days) (Heo et al., 2009)

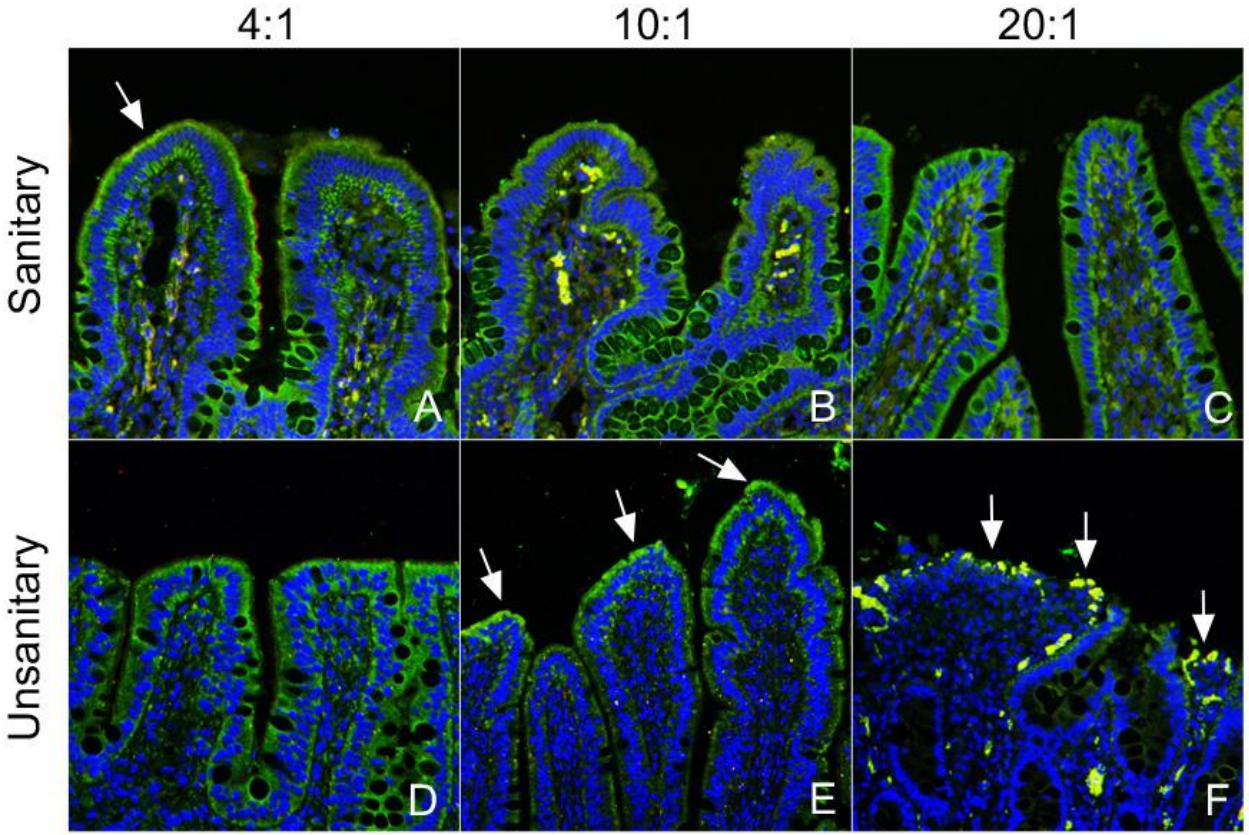

Fig. 1. Effect of sanitary challenge and dietary n-6:n-3 PUFA ratios on localization of tight junction protein occludin in the ileum of weaned pigs visualized using immunofluorescence technique. The localization of occludin (green) was shown in ileum sections [a white arrow (A-D); actin (red) and DNA (blue)], and disruptive patterns indicated in 4:1, 10:1 or 20:1 ratio of a poor sanitary condition [white arrows (E and F)]

Table 4. Effects of sanitary challenge and dietary n-6:n-3 PUFA ratios on occludin localization in weaned pig for 14 days after weaning

Item	Environmental conditions		SEM[1]	n-6:n-3 ratio			SEM	P-value[2]		
	Sanitary	Poor sanitary		20:1	10:1	4:1		E[3]	D[4]	E × D[5]
Occludin localization[6], %	49.4	76.8	7.23	72.2	57.0	60.2	2.02	***	†	NS

[1]Pooled standard error of mean

[2]Significance level: NS: Not significant, † P<0.1, * P<0.05, ** P<0.01, *** P<0.001

[3]Environmental condition (sanitary vs poor sanitary)

[4]Dietary treatment

[5]Interaction between dietary treatment and environmental condition

[6]Occludin localization criteria was expressed as the mean occludin localization (diffusion) value of pigs having score 1 (30.3%), score 2 (60.6%), and score 3 (99.9%)

Table 5. Effects of sanitary challenge and dietary n-6:n-3 PUFA ratio on circulating pro-inflammatory cytokines and lipid mediators at d 14 after weaning

Item	Environmental conditions		SEM[1]	n-6:n-3 ratio			SEM	P-value[2]		
	Sanitary	Poor sanitary		20:1	10:1	4:1		E[3]	D[4]	E × D[5]
Day 7										
IL-1β, pg/mL	0.0	12.0	3.58	18.0	0.0	0.0	8.69	†	NS	NS
TNF-α, pg/mL	13.5	16.4	4.98	16.8	14.6	13.4	3.11	NS	NS	NS
COX-2, pg/mL	191.0	318.7	64.28	295.3	257.3	211.9	59.87	*	NS	NS
PGE_2, pg/mL	35.8	125.2	41.25	90.5	88.8	62.3	25.32	***	**	NS
LTB_4, pg/mL	184.2	246.9	31.28	235.6	216.4	194.7	48.74	*	NS	NS
Day 14										
IL-1β, pg/mL	80.9	146.8	60.85	234.2	90.3	17.2	50.74	NS	*	**
TNF-α, pg/mL	23.9	35.6	5.24	35.8	29.8	24.3	4.21	*	†	NS
COX-2, pg/mL	1277.5	1845.6	257.14	1768.5	1604.6	1311.5	228.79	*	†	NS
PGE_2, pg/mL	416.4	646.6	44.23	634.5	601.2	358.8	39.54	**	**	*
LTB_4, pg/mL	458.4	715.1	72.04	659.7	589.2	511.5	78.24	**	**	NS

[1]Pooled standard error of mean

[2]Significance level: NS: Not significant, † P<0.1, * P<0.05, ** P<0.01, *** P<0.001

[3]Environmental condition (sanitary vs poor sanitary)

[4]Dietary treatment

[5]Interaction between dietary treatment and environmental condition

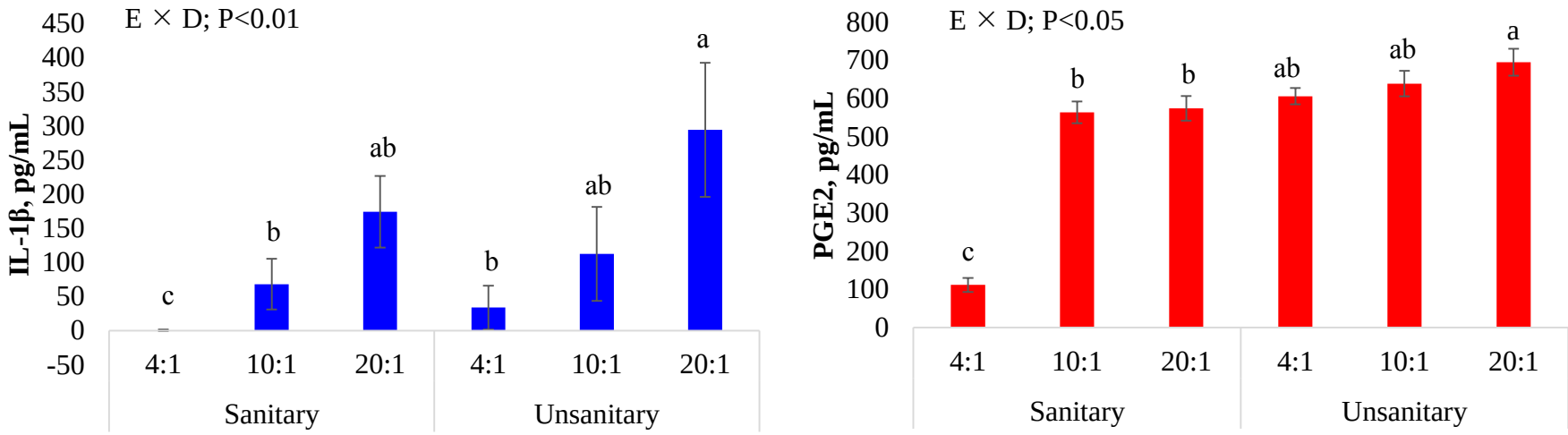

Figure 2. Interaction effects of sanitary challenge and dietary n-6:n-3 PUFA ratio on IL-1β and PGE_2 concentration on d 14 after weaning.

Interaction between environmental condition and dietary treatments were found for IL-1β and PGE_2 ($P<0.01$ and $P<0.05$, respectively).