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Early-life dietary spray-dried plasma influences immunological and intestinal injury responses to later-life *Salmonella typhimurium* challenge

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Abstract

Increasing evidence supports the concept that early-life environmental influences, including nutrition and stress, have an impact on long-term health outcomes and disease susceptibility. The objective of the present study was to determine whether dietary spray-dried plasma (SDP), fed during the first 2 weeks post-weaning (PW), influences subsequent immunological and intestinal injury responses to *Salmonella typhimurium* challenge. A total of thirty-two piglets (age 16–17 d) were weaned onto nursery diets containing 0, 2·5 % SDP (fed for 7 d PW) or 5 % SDP (fed for 14 d PW), and were then fed control diets (without SDP), for the remainder of the experiment. At 34 d PW (age 50 d), pigs were challenged with 3×10^9 colony-forming units of *S. typhimurium*. A control group (non-challenged) that was fed 0 % SDP in the nursery was included. At 2 d post-challenge, the distal ileum was harvested for the measurement of inflammatory, histological and intestinal physiological parameters. *S. typhimurium* challenge induced elevated ileal histological scores, myeloperoxidase (MPO), IL-8 and TNF, and increased intestinal permeability (indicated by reduced transepithelial voltage (potential difference) and elevated 4 kDa fluorescein isothiocyanate dextran (FD4) flux rates). Compared with *S. typhimurium*-challenged controls (0 % SDP), pigs fed the 5 % SDP-14 d diet exhibited reduced ileal histological scores, MPO levels, IL-8 levels and FD4 flux rates. Pigs fed the 5 % SDP-14 d nursery diet exhibited increased levels of plasma and ileal TNF-α in response to the challenge, compared with the other treatments. These results indicate that inclusion of SDP in PW diets can have an influence on subsequent immunological and intestinal injury responses induced by later-life *S. typhimurium* challenge.

Key words: Spray-dried plasma: Early-life nutrition: *Salmonella typhimurium*: Intestinal inflammation: Mucosal immunity: Intestinal permeability: Weaning

The gastrointestinal (GI) tract continues to undergo significant developmental changes in postnatal life. Environmental influences during this critical developmental period, including diet, stress and mucosal injury, have been shown to induce long-term changes in intestinal physiology and disease susceptibility in animal models[1–4]. Similarly in human subjects, increasing epidemiological evidence supports the concept that adverse early-life environmental factors, such as stress, are associated with subsequent GI diseases such as irritable bowel syndrome[5–9]. In the case of pigs, early (age <21 d) weaning of piglets is a significant, early-life stress that has been shown to have deleterious impacts on GI function, including increased intestinal permeability[10,11], inflammation[12], hypersecretion[10], reductions in the activity of brush-border digestive enzymes[13], altered nutrient transport mechanisms[14,15], and marked changes in villus and crypt morphology (reduced villus surface area and increased crypt depth)[16]. The mechanisms and factors associated with weaning stress (e.g. maternal and littermate separation, dietary changes, and transport stress) are not completely understood yet; however, it was demonstrated by Moeser *et al.*[10] that activation of the corticotropin-releasing factor (CRF) receptor system in the intestine, and subsequent activation of mast cells, were responsible for increased intestinal permeability and hypersecretion, demonstrating the role of stress signalling pathways in the intestine of the weaned pig. It is now evident that

Abbreviations: BW, body weight; CRF, corticotropin-releasing factor; ETEC, enterotoxigenic *Escherichia coli*; FD4, 4 kDa fluorescein isothiocyanate dextran; GI, gastrointestinal; MPO, myeloperoxidase; PD, potential difference; PW, post-weaning; SDP, spray-dried plasma; TBS-T, Tris-buffered saline with 0·1 % Tween-20; TER, transepithelial electrical resistance.

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the deleterious effects of early weaning stress on the intestinal tract of the pig are seen well beyond the immediate post-weaning (PW) period. Smith *et al.*[17] demonstrated that early-weaned pigs (weaned between 15 and 21 d of age) exhibited greater intestinal permeability at 9 weeks PW, compared with late-weaned pigs (weaned between 23 and 28 d of age). In addition, McLamb *et al.*[1] showed that early-weaned pigs exhibited heightened clinical disease (increased severity of diarrhoea and reduced growth rate) and intestinal injury (increased intestinal permeability), in response to an enterotoxigenic *Escherichia coli* (ETEC) challenge at approximately 3 weeks PW. On the whole, results from the aforementioned studies provide strong evidence that PW intestinal injury can have lasting deleterious impacts on intestinal function. Therefore, therapeutic approaches to ameliorate GI injury during the PW period could have a positive impact on long-term barrier function and defence against subsequent pathogenic challenges.

Dietary inclusion of spray-dried plasma (SDP) proteins in nursery pig diets has proven to have a beneficial effect on PW gastrointestinal health and performance in young pigs[18,19]. Previous studies demonstrated that SDP not only promotes growth responses in young pigs, but also confers protective effects in GI infectious challenge models. Van Dijk *et al.*[20] demonstrated that weaned pigs challenged with K88 ETEC, and fed a nursery diet containing 8 % SDP, exhibited reduced diarrhoea and increased average daily gain and average daily feed intake, compared with pigs fed control diets containing whey protein. In another experiment, weaned pigs fed with diets containing 6 % SDP exhibited reduced cytokine responses and intestinal inflammatory cell infiltrates, following a challenge with ETEC[21]. Similarly, diarrhoeal disease induced by an experimental rotavirus challenge has been observed to be reduced in neonatal piglets fed a diet containing 15 % SDP compared with control diets containing soya protein isolate[22]. Peace *et al.*[18] confirmed that inclusion of SDP at dietary levels of 2·5 and 5 % for 2 weeks PW reduced intestinal permeability, intestinal inflammatory cytokines and diarrhoea in early-weaned pigs. However, in the aforementioned previous experiments, growth responses and intestinal protective effects of SDP described were measured while SDP was in the diet; whether inclusion of SDP in early-life pig diets retains beneficial effects after its removal from the diet has not been investigated. Given that early-weaning stress induces short- and long-term deleterious changes in intestinal function and disease susceptibility, and that SDP has proven beneficial in reducing early changes in intestinal permeability and inflammatory responses in weaned pigs, we hypothesised that inclusion of SDP in PW pig diets would have sustained beneficial effects on intestinal responses to a later-life pathogenic challenge, even after the removal of SDP from the diet. The specific objective of the present study was to determine whether the inclusion of SDP during the first 2 weeks PW influenced intestinal epithelial barrier function, immune responses and clinical disease in response to a later-life challenge with *Salmonella typhimurium*.

Materials and methods

All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol no. 12-051-A).

Pigs and experimental design

A total of thirty-two Yorkshire–Large White piglets weaned from 16 to 17 d of age with a similar body weight of 5·49 (SEM 0·1) kg were used in the present experiment. The weaned piglets were housed in four nursery pens (eight pigs per pen; 1·09 m^2/pig) and were offered *ad libitum* access to water and one of three experimental nursery diets containing either 0 % SDP (fed to two pens, *n* 16 pigs), 2·5 % SDP (fed for 7 d PW; *n* 8 pigs) or 5 % SDP (fed for 14 d PW; *n* 8 pigs) (Fig. 1). Sex and litter origin were distributed equally across the experimental groups. The variable dietary levels of 2·5 and 5 % SDP along with feeding duration PW (7 *v.* 14 d PW) were selected to mimic the range of dietary level and feeding duration of SDP commonly utilised in commercial swine feeding. Diets were supplied in mashed form, and were formulated to contain identical levels of metabolisable energy and digestible lysine to meet nutrients requirements of the NRC (1998)[11]. At 7 d PW, pigs fed with the 2·5 % SDP treatment were switched to control (0 % SDP) diets. At 14 d PW, all pigs were fed the same diet (0 % SDP), and maintained in the nursery for an additional 21 d.

Salmonella typhimurium challenge

At 34 d PW, all pigs were transferred from the nursery to isolation rooms located in a nearby North Carolina State University research facility at the College of Veterinary Medicine campus. Upon arrival, the pigs were housed, by treatment, with eight pigs/pen (0·3 m^2/pig). The pens were equipped

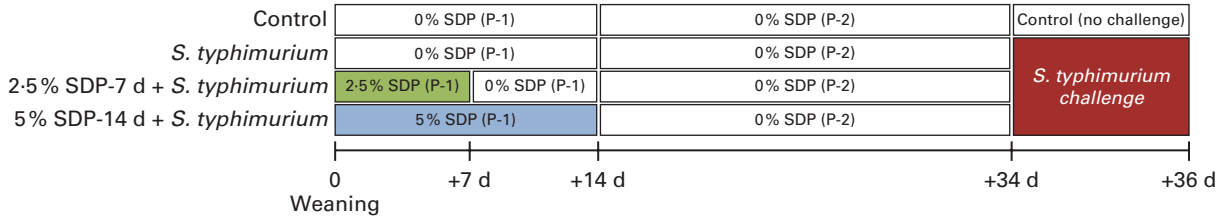

Fig. 1. Experimental design. Piglets (*n* 8 per treatment) were weaned from their sows and offered nursery diets containing either 0 % spray-dried plasma (SDP), 2·5 % SDP for 7 d post-weaning (PW) or 5 % SDP for 14 d PW. SDP was removed from the experimental diets at indicated times and fed identical diets to the control pigs. At 34 d PW, pigs were challenged with *Salmonella typhimurium*. At 2 d post-challenge, tissues were harvested for analysis. P-1, phase 1 diet; P-2, phase 2 diet. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

with tenderfoot flooring, and pigs were allowed *ad libitum* access to feed and water. On the following day, eight pigs from each experimental group were inoculated orally with 3×10^9 colony-forming units of *S. typhimurium* in 4 ml of culture media as described previously[23]. A non-challenged control group was housed in a separate, identical room within the facility, and was administered similarly with 4 ml sterile media. The *S. typhimurium* DT104 strain used in the present study exhibited antimicrobial resistance to ampicillin, chloramphenicol, sulfisoxazole, streptomycin and tetracycline. *Salmonella* cultures were grown overnight at 37°C on Luria broth agar, and then added to a sterile 0·7 % saline solution to obtain a final concentration of $7{\cdot}5 \times 10^8$ colony-forming units per ml, and verified using a NanoDrop 2000c nanospectrometer (Thermo Fisher Scientific, Inc.). In the present study, we chose *S. typhimurium* as the challenging agent because it has dual relevance to human and swine diseases[24].

Growth rate and feed intake calculations and faecal scores

Body weight (BW) was recorded at days 0 and 14 during the PW nursery phases, and on days 0 and 2 of the *S. typhimurium* challenge study, and average daily gain was calculated. Given the short (2 d) challenge period, growth data were presented as the percentage of BW loss. Pen feed intakes were recorded during the PW and *S. typhimurium* challenge periods, and estimated feed intake per pig was calculated for each pen. Faecal scores were analysed by persons, who were blinded to the experimental treatments, according to a previously published scoring system by our group[1] using a scale from 1 (no diarrhoea) to 4 (severe profuse diarrhoea).

Ussing chamber studies

On day 2 post-challenge, pigs were sedated with a TKX cocktail containing Telazol (500 mg), Ketamine (250 mg) and Xylazine (250 mg) that were administered intramuscularly at a dose of 0·025 ml/kg BW. Euthanasia was followed by the administration of an overdose (86 mg/kg BW) of sodium pentobarbital solution (Fatal-Plus; Virbac Animal Health) via a catheterised ear vein. The distal small intestine (ileum) was harvested from each pig immediately after euthanasia, and opened along the anti-mesenteric border. Intestinal mucosa was stripped from the seromuscular layer in oxygenated (95 % O_2 and 5 % CO_2) Ringer solution (in mmol/l: 154 Na^+, 6·3 K^+, 137 Cl^-, 0·3 H_2PO_4, 1·2 Ca^{2+}, 0·7 Mg^{2+} and 24 HCO_3^-; pH 7·4), and mounted in 1·13 cm^2 aperture Ussing chambers (World Precision Instruments, Inc.). Ileal mucosa was bathed on the serosal and mucosal sides with 10 ml Ringer solution. The serosal bathing solution contained 10 mM-glucose, which was osmotically balanced on the mucosal side with 10 mM-mannitol. Bathing solutions were oxygenated (95 % O_2 and 5 % CO_2) and circulated in water-jacketed reservoirs maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag–AgCl electrodes using a voltage clamp that corrected for fluid resistance. Tissues were maintained in the short-circuited state, except for brief intervals to record the open-circuit PD. Transepithelial electrical resistance (TER, measured as $\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}), as described previously[25]. After a 30 min equilibration period on Ussing chambers, TER and I_{sc} were recorded at 15 min intervals over a 1 h period, and then averaged to derive the basal TER and I_{sc} values for a given animal.

Paracellular permeability to 4 kDa fluorescein isothiocyanate dextran

After a 30 min equilibration period on Ussing chambers, 4 kDa fluorescein isothiocyanate dextran (FD4, 100 mg/ml; Sigma) was added to the mucosal bathing reservoir of Ussing chambers. Standards were taken from the serosal side of each chamber 15 min after the addition of FD4, and a 60 min flux period was established by taking 0·5 ml samples in triplicate from the mucosal compartment. The quantity of FD4 was established by measuring fluorescence in mucosal reservoir fluid samples in a fluorescence plate reader at 540 nm. Data are presented as the rate of FD4 flux in mg FD4 flux/min per cm^2.

Histological analyses of intestinal tissues

Ileum was fixed in 10 % neutral-buffered formalin and processed for paraffin embedding. Paraffin blocks were sectioned (5 μm thick) and stained with haematoxylin and eosin for histological analysis. A histological scoring system was applied to the tissue sections, and was performed by a board-certified veterinary pathologist (L. B. B.), who was blinded to the experimental treatments. The intestinal scoring system used was based on villus morphology and blunting (villus height and crypt depth), villus fusion, reduced lymphoid recruitment and neutrophil numbers. The detailed scoring criteria were designated as follows: villus blunting – 0 = crypt:tip ratio of at least 1:4, 1 = crypt:tip ratio of 1:3, 2 = 1:2, 3 = 1:1 and 4 = complete tip loss; lymphoid depletion and villus fusion – 0 = normal, 1 = mild, 2 = moderate and 3 = severe; neutrophils – 0 = none to 10 neutrophils/40 × field, 1 = 11–20 neutrophils/40 × field, 2 = 21–30 neutrophils/40 × field and 3 = 31–40 neutrophils/40 × field. Neutrophils were identified based on nuclear and cytoplasmic morphology[26]. Measurements for crypt depth and villus height were taken utilising the calibrated measurement caliper option, and villus measurements were taken from three well-oriented villi in five different fields/slide, such that fifteen villi/slide per pig were measured. Villi chosen for measurements were based on the criteria that (1) the entire crypt and villi be captured in the cross-section, and (2) the central lacteal be present. Villi overlying the gut-associated lymphoid tissue were excluded from the measurement. Photomicrographs were acquired with 20 × and 40 × magnifications at a resolution using imaging software (Infinity Analyze Software), running a high-resolution digital camera (Lumenera) equipped to a clinical light microscope (Model OMFL400; Meiji Microscope Solutions).

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Ileal cytokine analysis

Ileal mucosa was homogenised in PBS containing protease inhibitors, and the supernatant was collected and analysed for protein content using a BCA assay[18]. Samples were then diluted 1:10 in PBS and assayed for TNF, IL-8 and IL-6, using commercial porcine ELISA assays (R&D Systems). Concentrations of each cytokine are expressed on a per mg protein basis.

Myeloperoxidase assay

The distal ileum was obtained from each pig, opened lengthwise, and rinsed in cold Ringer solution. The epithelium and lamina propria were scraped from the seromuscular layers over ice using a glass slide, and then frozen in liquid N_2 and stored at −80°C. The ileal mucosal scrapings were thawed and homogenised in 0·5 % hexadecyltrimethylammonium bromide buffer (50 mM-phosphate buffer, pH 6), to release myeloperoxidase (MPO) from the primary granules of neutrophils. The homogenate was subjected to three cycles of freezing at −80°C, thawed, and sonicated on ice. Samples were centrifuged at 21 000 ***g*** at 4°C for 15 min, and the supernatant assayed for MPO activity. An aliquot of the supernatant was allowed to react with a solution of tetramethylbenzidine in *N*-dimethylformamide and H_2O_2. Absorbance (655 nm) readings were taken at 30 s intervals over 15 min. MPO activity was determined based on a MPO standard curve, and is expressed as units per g (wet weight) mucosa (for ileum) or per ml of plasma[27].

Western blot analysis of corticotropin-releasing factor receptors in the porcine ileum

Ileal mucosal protein was extracted from mucosal scrapes using Mammalian Protein Extraction Reagent containing protease and phosphatase inhibitors (Fisher Scientific). Samples were sonicated and centrifuged at 14 000 rpm for 15 min at 4°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Fisher Scientific). Total protein was resolved by SDS–PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5 % (w/v) non-fat milk in Tris-buffered saline with 0·1 % Tween-20 (TBS-T) for 1 h at room temperature, washed in TBS-T, and incubated with CRF-RI/II antibody that detects both receptors (Santa Cruz Biotechnology). Subsequently, the membranes were washed and incubated with an appropriate secondary antibody for 1 h at room temperature, followed by washing with TBS-T and incubation with the SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific). As an internal loading control, the antibody was stripped from the membranes with Restore™ Western blot stripping buffer (Thermo Fisher Scientific, Inc.), and the membranes were reprobed with a β-actin antibody (Cell Signaling Technology). Bands were visualised with ChemiDoc™ MP Imaging System (Bio-Rad), densitometric analysis was performed using the Bio-Rad Image Lab software (version 4.1), and the CRF receptor band intensities were normalised to β-actin.

Statistical analysis

Data are presented as means with their standard errors based on the experimental sample number (*n*). With the exception of histological and faecal score data, all other data were analysed using a standard one-way ANOVA (SigmaStat; Jandel Scientific). A *post hoc* Tukey's test was used to determine the differences between treatments following ANOVA. Statistical significance was set at a level of $P<0·05$. Histological and faecal scores were analysed using the non-parametric Kruskal–Wallis test (GraphPad Prism) with Dunn's post-test.

Results

Effects of early-life dietary spray-dried plasma on clinical responses to subsequent Salmonella typhimurium *challenge*

In the first 2 weeks PW, estimated feed intake for pigs fed diets containing 0, 2·5 % SDP (for 7 d) and 5 % SDP (for 14 d) was 0·221, 0·231 and 0·238 kg/d, respectively. The average daily gain (kg/d) during the first 2 weeks PW for pigs fed the 0, 2·5 and 5 % SDP diets was 0·121 (SEM 0·015), 0·119 (SEM 0·021) and 0·142 (SEM 0·025), respectively. All pigs remained clinically normal throughout the nursery phase. During the 2 d *S. typhimurium* challenge study, at 34 d PW, the control (non-challenged, 0 % SDP) pigs gained 5 % of their BW, whereas growth responses in pigs challenged with *S. typhimurium* were significantly reduced and ranged between 0·5 and −1 % BW gain (Fig. 2(A)). Compared with the non-challenged control pigs, the estimated feed intake of *S. typhimurium*-challenged pigs over the 2 d challenge period was 1·54, 1·20, 1·22 and 1·17 kg in pens from the control, 0 % SDP-, 2·5 % SDP- and 5 % SDP-challenged groups, respectively. All pigs challenged with *S. typhimurium* exhibited diarrhoea as indicated by higher ($P<0·05$) faecal scores, compared with the non-challenged control pigs (Fig. 2(C)). Rectal body temperatures were also significantly elevated in challenged pigs compared with the control ones ($P<0·05$; Fig. 2(B)). Dietary inclusion of SDP (2·5 or 5 % SDP) during the PW period had no significant effect on BW loss, faecal score or body temperature in response to *S. typhimurium* challenge in the present study.

Effects of early-life dietary spray-dried plasma on histological injury responses to Salmonella typhimurium *challenge*

Compared with the non-challenged control group, *S. typhimurium*-challenged pigs exhibited elevated ileal histological injury scores (Fig. 3(A)). Histological scores from pigs fed with the 5 % SDP-14 d diet during the nursery period were lower compared with those from the challenged control pigs. Marked lymphoid depletion, an index of an overwhelming immune response, was observed in all *S. typhimurium*-challenged pigs, but was less severe in pigs fed with the 2·5 %-7 d and 5 % SDP-14 d PW diets. Extensive villus blunting (Fig. 3(A) and (B)) and fusion (adhesion) was observed in all pigs challenged with *S. typhimurium*; however, there were no effects of PW SDP treatments on

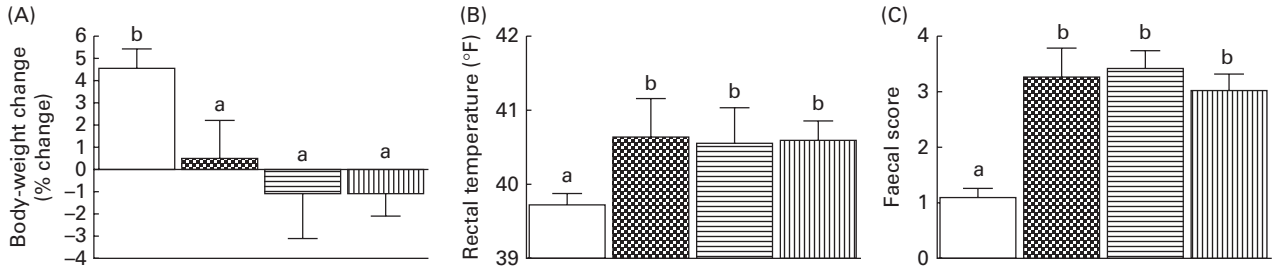

Fig. 2. Body weight, body temperature and faecal scores in pigs challenged with *Salmonella typhimurium*. (A) Body-weight loss was calculated from body weights recorded on day 0 and day 2 post-challenge. (B) Rectal temperature and (C) faecal scores were recorded on day 2 post-challenge. Values are means, with their standard errors represented by vertical bars (n 8). [a,b] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). SDP, spray-dried plasma. □, Control; ▩, *S. typhimurium*; ▤, 2·5 % SDP + *S. typhimurium*; ▥, 5 % SDP + *S. typhimurium*.

these parameters. Crypt depth was found to be increased ($P<0·05$) in the ileum from *S. typhimurium*-challenged pigs compared with the control ones (Fig. 3(C)). Ileum from pigs fed with the 5 % SDP-14 PW diet had increased ($P<0·05$) crypt depth compared with all the other treatments. Increased numbers of ileal neutrophils (Fig. 3(A)) were observed in response to *S. typhimurium* challenge, which corresponded with higher activity of ileal MPO, a marker of neutrophil activation (Fig. 4(A)). MPO and neutrophil numbers were lower in the ileum from pigs fed with 5 % SDP-14 d diet in the PW period.

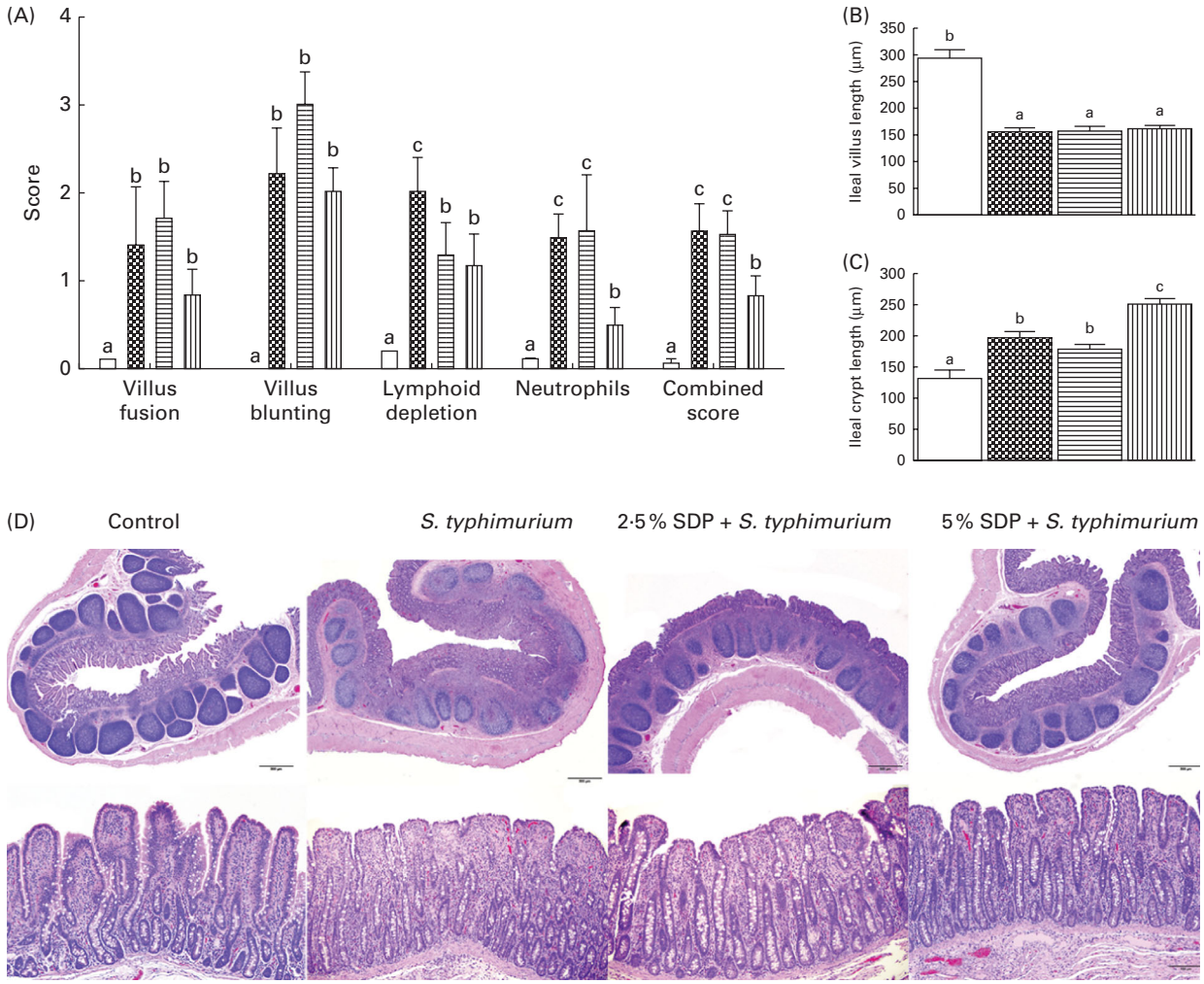

Fig. 3. Impact of early-life dietary spray-dried plasma (SDP) on histological damage caused by subsequent challenge with *Salmonella typhimurium*. (A) Histological scores, (B) villus height, (C) crypt length and (D) histological appearance from pig ileal tissues, at 2 d post-*S. typhimurium* challenge. Representative histological sections were taken at 4 × and 20 × magnification. Values are means, with their standard errors represented by vertical bars (n 8). [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). □, Control; ▩, *S. typhimurium*; ▤, 2·5 % SDP + *S. typhimurium*; ▥, 5 % SDP + *S. typhimurium*. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

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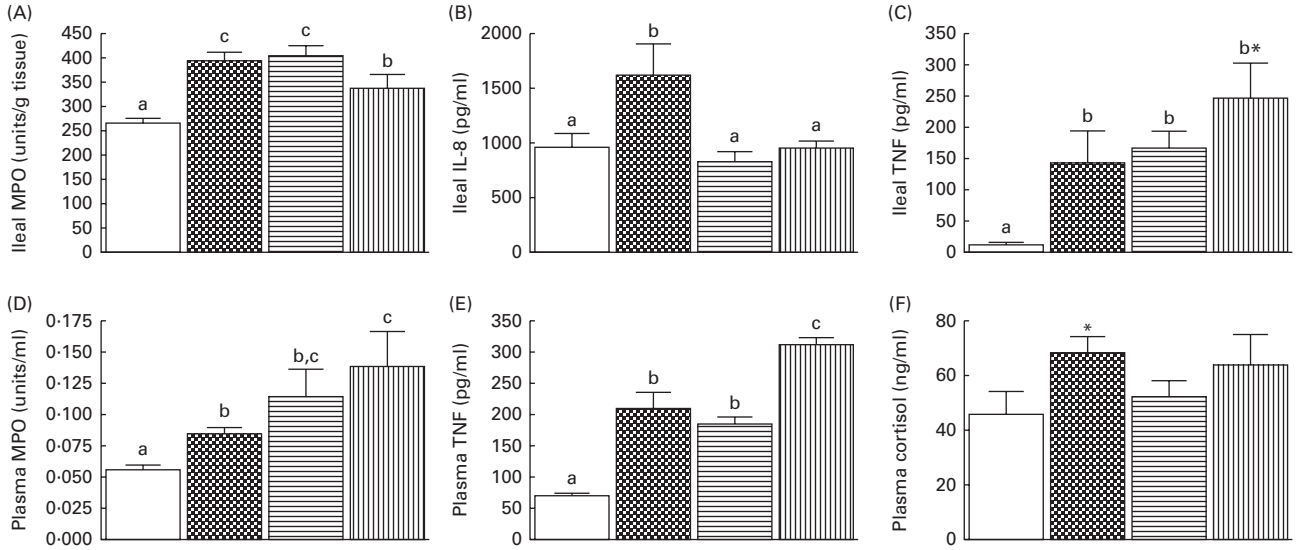

Fig. 4. Impact of early-life dietary spray-dried plasma (SDP) on ileal and plasma immunological responses to subsequent *Salmonella typhimurium* challenge. (A) Ileal myeloperoxidase (MPO), (B) ileal IL-8, (C) ileal TNF, (D) plasma MPO, (E) plasma TNF and (F) plasma cortisol were measured at 2 d post-*S. typhimurium* challenge. Values are means, with their standard errors represented by vertical bars (n 8). [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). * At trend for ileal TNF between 5 % SDP and the other challenged treatments ($P=0·07$) and for plasma cortisol between *S. typhimurium* control and control ($P=0·09$). □, Control; ⊠, *S. typhimurium*; ⊟, 2·5 % SDP + *S. typhimurium*; ▥, 5 % SDP + *S. typhimurium*.

Effects of spray-dried plasma on ileal and plasma cytokines in response to later-life Salmonella typhimurium *challenge*

TNF concentrations were found to be elevated in the ileum from all *S. typhimurium*-challenged groups compared with the non-challenged control group (Fig. 4(C)). There was a trend ($P=0·06$) for elevated TNF levels in response to *S. typhimurium* challenge in pigs fed the 5 % SDP-14 d diet, compared with the other challenged treatments. Ileal IL-8 levels were increased in the *S. typhimurium*-challenged group (Fig. 4(B)); however, IL-8 levels were lower in the ileum from the challenged groups fed the 2·5 %-7 d or 5 % SDP-14 d diet in the PW period (Fig. 4(B)).

To assess the effects of *S. typhimurium* infection and SDP nursery feeding on systemic inflammatory responses, plasma levels of MPO (Fig. 4(D)), TNF (Fig. 4(E)) and cortisol (Fig. 4(F)) were assessed 2 d post-challenge. Plasma TNF was elevated in all pigs challenged with *S. typhimurium*. In line with responses observed in the ileum, pigs fed with the 5 % SDP-14 d nursery diet exhibited the greatest levels of plasma TNF in response to *S. typhimurium* challenge. Plasma cortisol levels tended to be elevated ($P=0·09$) in the *S. typhimurium*-challenged control group, but were not different from the other experimental treatment groups.

Utilising an antibody that recognises both CRF receptor subtypes (CRF_1 and CRF_2), we found that the $CRF_{1/2}$ antibody recognised three major protein bands in porcine ileal protein extracts at approximately 55, 37 and 28 kDa (Fig. 5) . These protein bands are consistent with the unprocessed form (55 kDa), the deglycosylated form (37 kDa) and the soluble CRF receptor forms (28 kDa)[28], and have been reported previously in studies on the rodent intestine[29,30]. Based upon densitometric analysis, intestinal $CRF_{1/2}$ receptor proteins (50 and 28 kDa bands) were markedly up-regulated ($P<0·05$) in response to *S. typhimurium* challenge. However, the nursery SDP treatment did not appear to influence the level of ileal CRF receptor expression in the challenged pigs.

Effects of spray-dried plasma on intestinal permeability in response to later-life Salmonella typhimurium *challenge*

At 2 d post-challenge, FD4 flux rates, an index of paracellular permeability, were elevated ($P<0·05$) in the ileum from pigs challenged with *S. typhimurium* ($P<0·05$) (Fig. 6(A)). Pigs fed with the 5 % SDP-14 d diet during the PW period exhibited lower FD4 flux rates at 2 d post-challenge, compared with the other challenged treatment groups. Ileal TER was higher in the challenged pigs compared with the non-challenged control pigs ($P<0·05$; Fig. 6(B)). There was a trend ($P=0·06$) for increased ileal TER in pigs fed the 2·5 % SDP-7 d and 5 % SDP-14 d diets during the PW period compared with the challenged control pigs. Transepithelial PD and short-circuit current (I_{sc}) were reduced in *S. typhimurium*-challenged pigs compared with the non-challenged control pigs (Fig. 6(C) and (D), respectively). Pigs fed with the 5 % SDP-14 d diet exhibited greater ileal PD, compared with the challenged control pigs ($P<0·05$). Ileal I_{sc} was greater in pigs fed with the 2·5 % SDP-7 d and 5 % SDP-14 d diets compared with non-challenged control pigs.

Discussion

Inclusion of SDP into animal diets has been shown to promote growth responses[31–34], and lessen inflammatory processes and clinical disease in pathogen challenge models[20–22,35]. Previously, we demonstrated that dietary SDP, at 2·5 and 5 % of weaned pig diets, was beneficial in reducing intestinal

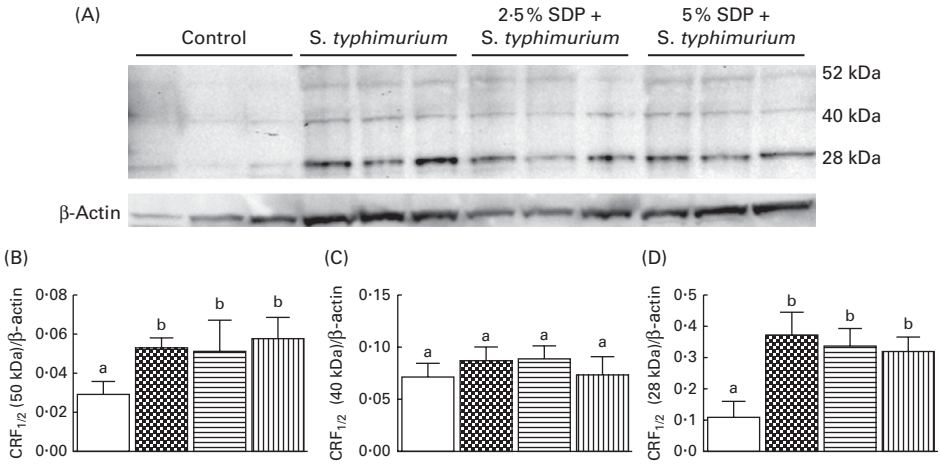

Fig. 5. (A) Western blot and (B) densitometric analysis of corticotropin-releasing factor ($CRF_{1/2}$) receptors (CRFR) in porcine ileal mucosal scrapes from control and *Salmonella typhimurium*-challenged pigs. (B–D) Densitometry values for each protein band normalised to β-actin are shown. Values are means, with their standard errors represented by vertical bars (n 3). [a,b] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). SDP, spray-dried plasma. □, Control; ▩, *S. typhimurium*; ▤, 2·5 % SDP + *S. typhimurium*; ▥, 5 % SDP + *S. typhimurium*.

permeability and inflammation induced in early-weaned pigs[18]. While the aforementioned studies on rodents and pigs demonstrated the beneficial effects of SDP on growth and intestinal inflammatory responses to stress and pathogenic challenges, the response variables were measured when SDP was currently being included in the diet. Whether or not SDP confers beneficial effects on the intestine after its removal from the diet has not been investigated. Here, we showed that dietary inclusion of SDP during the first 2 weeks PW can modify subsequent intestinal and immunological responses to a pathogenic challenge with *S. typhimurium* in pigs.

In the present study, pigs fed diets containing SDP during the first 2 weeks PW exhibited differential intestinal and systemic immune responses following an *S. typhimurium* challenge at 50 d of age; compared with the challenged control pigs, those fed the 2·5 % SDP-7 d and 5 % SDP-14 d diets during the PW period exhibited reduced ileal IL-8 levels. IL-8 is a major inflammatory cytokine produced by intestinal epithelial cells during *S. typhimurium* infection, and it acts as a chemoattractant for the recruitment of circulating neutrophils into the intestine, resulting in classic intestinal inflammatory lesions associated with *S. typhimurium* enteritis[36,37]. In line with this role,

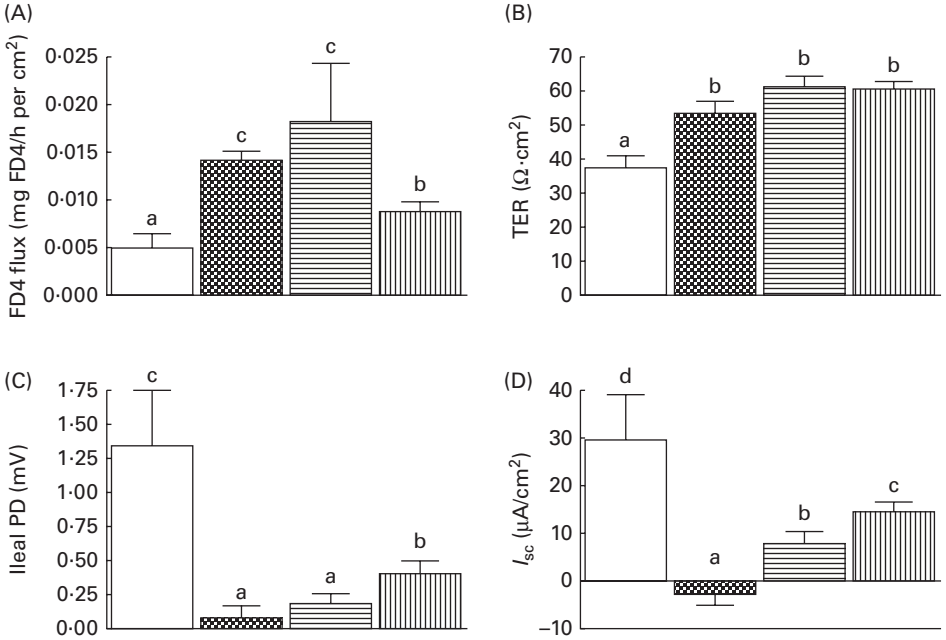

Fig. 6. Impact of early-life dietary spray-dried plasma (SDP) on intestinal permeability and transepithelial short-circuit current (I_{sc}) following subsequent *Salmonella typhimurium* challenge in pigs. (A) Fluorescein isothiocyanate dextran (FD4) flux rates, (B) ileal transepithelial electrical resistance (TER), (C) ileal transepithelial potential difference (PD), and (D) I_{sc} were measured using the ileum mounted on Ussing chambers on 2 d post-*S. typhimurium* challenge. Values are means, with their standard errors represented by vertical bars (n 8). [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). □, Control; ▩, *S. typhimurium*; ▤, 2·5 % SDP + *S. typhimurium*; ▥, 5 % SDP + *S. typhimurium*.

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pigs fed with the 5% SDP-14 d nursery dietary treatment had reduced neutrophil infiltration, MPO levels, and histological injury in response to *S. typhimurium* challenge. Bosi *et al.*[21] demonstrated earlier, in agreement with our findings, that piglets fed with 6% SDP exhibited reduced ileal IL-8 concentrations, induced by ETEC challenge; however, unlike the present study, IL-8 levels were measured while SDP was included in the diet at the time of the challenge.

Interestingly, despite the dampened histopathological and inflammatory responses exhibited by the challenged pigs fed with the 5% SDP-14 d nursery diet, ileal and plasma TNF levels were higher compared with the challenged control pigs. As mentioned above, *S. typhimurium* induces an intestinal inflammatory response mediated via the production of pro-inflammatory cytokines, including TNF and IL-8 and subsequent neutrophil recruitment and activation[38]. TNF is best recognised as a pro-inflammatory cytokine that is central to the pathogenesis of a number of inflammatory disorders, and to stress-induced intestinal permeability[39,40].

Also, TNF is recognised as a critical and beneficial modulator of immune function and pathogen defence. For example, Nauciel & Espinasse-Maes[41] demonstrated that administration of anti-TNF antibodies to mice exacerbated bacterial proliferation and mortality, following a sub-lethal dose of *S. typhimurium*. In similar studies carried out by Gulig *et al.*[42] and Tite *et al.*[43], anti-TNF antibodies increased the numbers of splenic colony-forming units of *S. typhimurium* following challenge.

Collectively, these studies have suggested that elevated TNF responses are critical for the control of infections. The present study is consistent with the study by Touchette *et al.*[44] showing that early-weaned pigs fed with a diet containing 7% SDP for 7 d PW exhibited a 2-fold higher increase in serum TNF levels in response to systemic LPS challenge, compared with pigs that did not receive SDP. The authors also demonstrated a marked 110-fold increase in (interferon-α) in pigs, fed with the SDP-enriched diet, in contrast to a 16-fold increase in pigs fed a diet without SDP. Taken as a whole, the present study, along with the previous investigations, demonstrated that dietary SDP can modulate local and systemic immune responses to weaning and pathogen challenges. However, the present study provides, for the first time, evidence that the effects of SDP on immune responses can be retained even after the removal of SDP from the diet.

In addition to investigating the effects of early dietary SDP and subsequent *S. typhimurium* challenge on inflammatory signals, we also investigated stress signalling pathways. Specifically, we showed that plasma cortisol and intestinal expression of CRF receptors were increased 2 d post-challenge; however, there were no differences between pigs fed SDP. While elevations in plasma cortisol, following *S. typhimurium* challenge, have been shown previously in studies on pigs[23], our findings on the marked up-regulation of intestinal $CRF_{1/2}$ receptors during an acute challenge with S. *typhimurium* are novel. Given the increasingly recognised role of the intestinal CRF system in inflammatory and stress-induced functional GI disorders, in human subjects and laboratory research animal models[17,45,46], these findings warrant further investigation into the role of CRF in infectious inflammatory diseases.

In the present study, *S. typhimurium* challenge induced impairment in intestinal barrier function, which was indicated by increased ileal permeability to the paracellular probe FD4. The increase in ileal permeability in *S. typhimurium*-challenged pigs was attenuated in pigs fed with the 5% SDP-14 d nursery diet, suggesting either an protective or reparative influence of early SDP dietary inclusion on intestinal barrier function.

It is not understood how early SDP feeding resulted in lasting protective effects on intestinal barrier during *S. typhimurium* infection in the present study. However, it is known that intestinal neutrophil infiltration in response to *S. typhimurium* infection is a central process, contributing to the breakdown of intestinal barrier function. Neutrophil-mediated disruption of intestinal barrier function involves a multi-step mechanism, including increased myosin light chain phosphorylation and myosin light chain kinase, up-regulation of tight junction phosphotyrosine and phopsho-serine residues[47], and activation of epithelial protease-activated receptors[48]. Given that the 5% SDP-14 d inclusion in nursery diets resulted in reduced neutrophil infiltration in response to *S. typhimurium* infection, it is plausible that this may represent an important mechanism, by which SDP led to a protective effect on the intestinal barrier in the present study.

In addition, we observed unexpected results with regard to ileal TER in the present study. Despite the elevated FD4 permeability in the ileum at 2 d post-challenge, ileal TER was significantly elevated in all the challenged groups. FD4 flux rates and TER measure two different aspects of intestinal epithelial barrier function: TER reflects changes in ion (predominantly Na^+) permeability across the tight junction pores, while FD4 flux reflects large molecule fluxes across the leaky tight junctions. Another difference between the two measurements is that TER is calculated, on the basis of measured values of transepithelial voltage (PD) and current (I_{sc}), according to Ohm's law ($V = IR$), and expressed on the basis of the surface area of the tissue chamber aperture. Therefore, significant alterations in either PD or I_{sc} could have a significant impact on calculated TER values. Further analysis of PD across *S. typhimurium*-infected ileum tissues revealed a significant reduction in PD, which indicates a compromised ability of the intestinal epithelium to resist ion flow through the paracellular space, and thus is in line with the elevated FD4 flux. However, in contrast to the FD4 flux data, PD was not significantly influenced by the early nursery 5% SDP-14 d dietary treatment. *S. typhimurium* challenge also resulted in significant reductions in ileal I_{sc}, which, in turn, might have contributed to the increased calculated TER values. Furthermore, I_{sc} was greater in pigs fed the 2·5%-7 d and 5% SDP-14 d nursery diets, which accounted for the increased TER in pigs fed the SDP treatments. The basis for increased I_{sc} observed in the challenged pigs fed SDP in the nursery is not clear. However, the suppressive influence of *S. typhimurium* on I_{sc} has been demonstrated in previous investigations on pigs and mice[49,50]. The mechanisms for reduced I_{sc} in the ileum from *S. typhimurium*-challenged pigs could be due to reduced anion (Cl^- or HCO_3^-) secretion

or electrogenic cation (e.g. Na^+) absorption. In a recent study, it has been demonstrated that mice challenged with *S. typhimurium* exhibited reduced basal- and adenosine 3′,5′-cyclic monophosphate-mediated electrogenic I_{sc}, an effect associated with reduced expression and/or localisation of colonic epithelial ion transporters including the Cl^-/HCO_3^- exchanger down-regulated in adenoma and the cystic fibrosis transmembrane regulator[51]. These suppressive effects were, in part, mediated by secreted *S. typhimurium* effector proteins. Therefore, in the light of these findings, it is plausible that the influence of early SDP on subsequent I_{sc} responses to *S. typhimurium* challenge could be directly related to the effects of SDP treatments on subsequent *S. typhimurium* pathogenicity in the porcine intestine. The precise host intestinal pathways modulated by early SDP feeding in pigs that contribute to the I_{sc} response remain to be elucidated.

Despite marked changes in immune and epithelial barrier responses, SDP had little influence on *S. typhimurium*-induced morphology of intestinal villi (villus blunting or villus fusion) and epithelium (denuded epithelium). Interestingly, increased crypt depths were observed in pig fed the 5% SDP-14 d nursery diet. Increased crypt depth (crypt expansion) is a hallmark of intestinal injury, but at the same time is an index of epithelial repair processes, as increased proliferation of immature crypt enterocytes will migrate to the villus tip to replace damaged or denuded villus epithelial cells. Therefore, the increased crypt depths in pigs fed the 5% SDP nursery diet could indicate increased epithelial renewal, which might potentially prove beneficial in later stages of recovery from *S. typhimurium*.

As mentioned previously, there are a number of studies in the literature that describe the beneficial impact of dietary SDP on growth responses. However, in the present study, there were no significant differences in growth and/or clinical responses in pigs observed either during the PW period or during the subsequent *S. typhimurium* post-challenge period. Despite the lack of measurable growth response to SDP in the present study, significant effects on immunological and intestinal responses were demonstrated.

There are several reasons that could explain the lack of SDP growth responses in the present study. First, the primary objective was not to measure growth performance, but to determine whether early dietary SDP influenced subsequent immunological and intestinal physiological responses to a later-life *S. typhimurium* challenge. Therefore, sufficient animal sample size needed to achieve the statistical power required to appropriately evaluate growth responses was not included in the experimental design.

Second, the experimental environment in which the pigs were raised might not have been ideal to demonstrate a SDP-dependent growth response. It has been shown previously that the effects of SDP on pig growth were observed in a commercial farm environment, but not in an experimental university research setting[34]. A third reason for the lack of SDP growth response, specifically observed in the post-challenge phase of the experiment, is the short time period (2 d) in which BW changes were measured, which might have been inadequate to assess post-challenge growth responses during the peak challenge response. Given the beneficial effects of early SDP observed on intestinal physiological and immunological responses following *S. typhimurium* challenge, growth measurements over a longer post-challenge period (e.g. 7–14 d) could have a significant influence on the effects of SDP on growth responses in challenged pigs.

In conclusion, data from the present study demonstrate that early dietary inclusion of SDP have an impact on intestinal immunological and epithelial pathophysiological responses to *S. typhimurium* challenge, even after SDP has been removed from the diet. Given that stress and diet are increasingly recognised as key early-life factors that determine long-term health outcomes in human subjects and animals, a more fundamental understanding of biological mechanisms and optimal nutritional intervention strategies, such as dietary SDP, have potential to exert a positive impact on long-term intestinal health.

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None of the authors has any conflict of interest to declare.

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