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Research paper

Microalgae supplementation to late gestation sows and its effects on the health status of weaned piglets fed diets containing high- or low-quality protein sources

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ABSTRACT

Maternal stress, such as a bacterial infection occurring in late gestation, may predispose offspring to a variety of diseases later in life. It may also alter programming of developing systems within the fetus, such as the hypothalamic-pituitary-adrenal (HPA) axis and immune system. Dietary supplementation during the last trimester of pregnancy with immune-modulating compounds may be a means of reducing potential adverse effects of maternal stress on the developing fetus. Essential omega-3 polyunsaturated fatty acids (n-3 PUFA) such as docosahexanoic acid (DHA) and eicosapentanoic acid are well-known for their immune-modulating and anti-inflammatory properties. Sources of these n-3 PUFA include fish products such as fish oil and microalgae, which may be a suitable alternative to fish-based products. The aim of this study was to determine the effect of supplementing gestating sow diets with n-3 PUFA and inducing an immune stress challenge in late gestation on piglet growth and immune responsiveness when placed on either a high- or low-quality protein diet after weaning. Forty-eight sows were fed gestation diets containing either 3.12% microalgae, 3.1% fish oil or a corn oil control diet containing 1.89% corn oil starting on gestation day (gd) 75. On gd112, half the sows in each treatment were immune stress challenged with bacterial lipopolysaccharide (LPS) endotoxin (10 μg/kg administered *i.m*). After farrowing, piglet BW gain was monitored weekly during lactation and pigs were weaned at 21 days of age. One week after weaning, four piglets per sow were immune stress challenged with LPS (40 μg/kg administered *i.m.*). At the same time, four piglets per sow were vaccinated with the novel antigens chicken ovalbumin (OVA) and *Candida* cellular antigen (CAA) and received booster vaccinations two weeks later. Four weeks after the initial vaccination, a transdermal hypersensitivity immune challenge was performed using the same antigens. Blood samples were also collected to quantify IgG antibody responses to both antigens. PUFA enrichment in sow blood and piglet brain was detected after sows were on feed for 40 days. Piglet growth was increased in pigs fed a high-quality diet in nursery phase 1. Concentrations of the cytokines IL-1ra, IL-6 and IL-10 were elevated in pigs fed a high-quality protein diet following LPS immune challenge. Overall, it appears that in the current study piglet nursery diet quality was more important for determining piglet health and growth than maternal diet and immune stress.

1. Introduction

Maintaining piglet health is important around weaning to promote growth and maximize the efficiency of the animal. At weaning, piglets are suddenly stressed as they are removed from their dams and placed into an environment with a potentially high pathogen load (Campbell et al., 2013; Martínez-Miró et al., 2016). In addition, their immune system is not fully developed at this time, and they therefore rely on maternal antibodies for immunity (Campbell et al., 2013). Stress encountered early in life, including microbial infection, can compromise piglet growth and immune function and can affect the long-term health of the animal (Campbell et al., 2013).

To help newly weaned pigs reach their growth potential and mount efficient and appropriate immune responses, the industry standard is to include high-quality, expensive protein sources such as whey protein or purified protein isolates to the diets of newly weaned pigs (Goodband

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Table 1
Analyzed concentrations of EPA, DHA, Total n-3 and ratio of n-3:n-6 in sow plasma, colostrum and fetal brain from sows fed diets supplemented with 1.26% (n = 4) or 3.12% microalgae (AL; n = 4), 1.25% (n = 4) or 3.1% fish oil (FO; n = 4), or a corn oil (CO; n = 4) control diet. Data are presented as LSM +/- SEM.

Sow diet	CO[1]	1.26% AL[2]	3.1% AL	3.1% FO[3]	1.25% FO	SEM[4]	P-value[5]
Plasma n = 4 (mg/g)							
EPA[6]	0.21^{c}	1.30^{c}	2.50^{cb}	8.96^{a}	4.95^{b}	0.74	< 0.0001
DHA[7]	0.00^{d}	5.46^{b}	8.10^{a}	4.36^{b}	2.68^{c}	0.41	< 0.0001
Total n-3[8]	2.87^{c}	9.63^{b}	13.37^{b}	18.48^{a}	12.17^{b}	1.22	< 0.0001
Ratio n3:n6[9]	0.06^{d}	0.17^{c}	0.24^{b}	0.34^{a}	0.20^{bc}	0.01	< 0.0001
Colostrum n = 4 (mg/100 g)							
EPA	4.44^{c}	15.07^{c}	18.46^{c}	128.36^{a}	57.42^{b}	6.99	< 0.0001
DHA	2.37^{ac}	79.40^{ab}	149.81^{b}	108.48^{b}	43.21^{c}	22.42	0.0091
Total n-3	85.16^{b}	178.30^{b}	240.24^{ab}	436.73^{a}	257.06^{ab}	54.78	0.0090
Ratio n3:n6	0.05^{c}	0.12^{b}	0.20^{a}	0.23^{a}	0.15^{b}	0.01	< 0.0001
Fetal brain n = 2 (mg/100 g)							
EPA	1.95^{b}	6.77^{b}	18.70^{b}	33.32^{a}	16.23^{ab}	5.35	0.0091
DHA	1053.17^{b}	1535.41^{a}	1801.62^{a}	1710.18^{a}	1496.01^{a}	100.91	0.0041
Total n-3	1151.59^{b}	1663.42^{a}	1993.11^{a}	1929.49^{a}	1621.16^{a}	105.33	0.0020
Ratio n3:n6	0.33^{c}	0.67^{b}	0.76^{bc}	0.86^{a}	0.63^{b}	0.04	< 0.0001

^{a,b,c,d}Differing letters across rows indicate significant differences among treatments ($P < 0.05$).

[1] CO, Sow diet containing 1.89% corn oil as a control.
[2] AL, microalgae.
[3] FO, fish oil.
[4] SEM, maximum value of the standard error of the means.
[5] P-value for the main effect of dietary treatment.
[6] EPA, eicosapentanoic acid.
[7] DHA, docosahexanoic acid.
[8] Total n-3, total omega-3 polyunsaturated fatty acids.
[9] Ratio n-3:n-6, ratio of total omega-3 polyunsaturated fatty acid to total omega-6 polyunsaturated fatty acids.

et al., 2014). However, researchers have explored the possibility of including simple, less expensive protein sources such as soybean or canola meals in weanling pig diets to reduce feed costs without compromising growth or health (Collins et al., 2017; Skinner et al., 2014).

Sows are sensitive to stress, and environmental and social changes as well as microbial infections during gestation can disrupt pregnancy and result in abortion, increase number of stillbirths, and/or decrease litter sizes (Peltoniemi et al., 2016). The stress response, mediated by the hypothalamic-pituitary-adrenal (HPA) axis, is activated during Gram-negative microbial infections (Martínez-Miró et al., 2016; Veru et al., 2014) by cell wall lipopolysaccharide (LPS). Activation of the HPA axis produces several signalling neuropeptides and hormones, ultimately resulting in the production of cortisol from the adrenal glands.

The production of maternal cortisol can have negative effects on the developing fetus *in utero*. Maternal cortisol can cross into fetal circulation despite placental protective mechanisms (Marques et al., 2015). Fetal exposure to maternal stress during gestation can negatively affect offspring growth, stress responsiveness, and immune regulation and function (Götz et al., 2007; Marques et al., 2015; Solano et al., 2016). Limiting the effects of maternal stress on the developing offspring may therefore be an important for ensuring normal piglet health and preventing the occurrence of disease in the peri-weaning period.

Supplementing the maternal diet with immune-modulating compounds such as omega-3 polyunsaturated fatty acids (n-3 PUFA) may be beneficial during pregnancy as a way to reduce inflammation and limit potential adverse effects of maternal cortisol on fetal development (Carroll et al., 2003; Fisher et al., 2014; Liu et al., 2013). Omega-3 PUFA, known for their anti-inflammatory properties, have been shown to affect the differentiation, trafficking and activity of immune cells, decreasing inflammation and favoring tissue resolution (Calder, 2013; Fisher-Heffernan et al., 2015). While acute inflammation may be beneficial, chronic inflammation can contribute to tissue pathology and compromise tissue function (Dhabhar, 2014). Thus, inclusion of n-3 PUFA in maternal diets at an optimal n-6 to n-3 PUFA ratio may be a way of modulating offspring immunity and providing enhanced protection against pathogens that contribute to inflammatory disease.

Most dietary sources of n-3 PUFA are fish-based; however, there is growing interest in finding alternative sources of n-3 PUFA. A promising source of n-3 PUFA is microalgae, which can be grown in tightly controlled conditions (Robertson et al., 2015; Yaakob et al., 2014) and can be rich in either docosahexanoic acid (DHA), eicosapentanoic acid or both, depending on the species (Singh and Saxena, 2015). As algae products become better characterized, they may also become an economical source of n-3 PUFA for livestock diets. While the use of supplements containing n-3 PUFA in sow diets has been explored (Rooke et al., 1998; Shen et al., 2015), there is limited information about the use of microalgae as a source of n-3 PUFA in sow diets and the subsequent effects on the offspring (Gázquez et al., 2017; Posser et al., 2018). Therefore, this study aimed to assess the stress and acquired immune responses of offspring from sows supplemented with dietary fish oil, microalgae or corn oil and challenged with LPS endotoxin in late gestation, when these piglets were weaned onto a high- or low-quality protein nursery diet.

2. Methods

The study was conducted at the Arkell Swine Research Station at the University of Guelph (Guelph, ON, Canada). The vitamin E and the Menhaden fish oil for this experiment were provided by Grand Valley Fortifiers (Cambridge, ON) and the microalgae (*Aurantiochytrium limacinum* biomass [AURA; CCAP 4087/2] containing 70% crude fat and 17% DHA) was provided by Alltech Inc. (Nicholasville, KY). The experimental protocol (AUP # 3124) was approved by the University of Guelph Animal Care Committee and followed Canadian Council of Animal Care guidelines (CCAC, 2009). The experimental diets were analyzed for dry matter, crude protein and macro-mineral content by SGS Canada (Guelph, ON) (Table 1).

2.1. Sow feeding trial and LPS immune stress challenge

For the sow feeding trial (Fig. 1), 48 Landrace x Yorkshire crossbred sows (12 sows per block × 4 blocks) were selected based on breeding date and parity (≥2) and were randomly assigned to one of three gestation dietary treatments: 3.1% fish oil, 3.12% microalgae, or a 1.89%

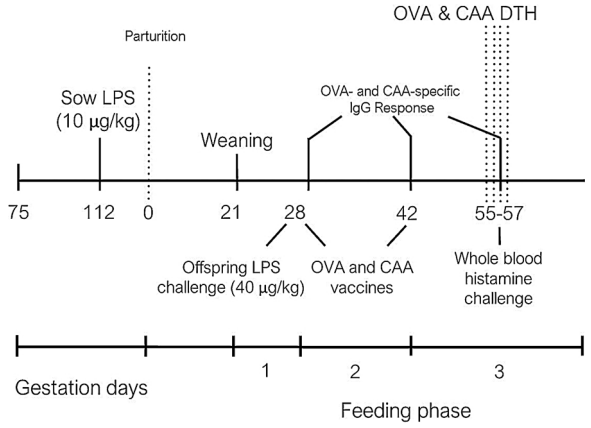

Fig. 1. Schematic time line of events for sow and piglet trial. LPS — lipopolysaccharide, DTH — delayed-type hypersensitivity, OVA — Ovalbumin, CAA — candida albicans antigen, IgG — Immunoglobulin G.

corn oil control diet. Sows were fed the assigned gestation diets, 2.5 kg of diet per day, from gd75 until farrowing (approx. gd 114), and were given unlimited access to fresh water. The experimental diets were formulated to meet the estimated nutrient requirements for sows in late gestation (National Research Council, 2012) and were formulated to be isonitrogenous and isocaloric. The experimental microalgae and fish oil diets were also formulated to have similar DHA content. Ingredient composition of dietary treatments are shown in Table 2.

A pilot study determined that an intramuscular injection with 10 µg LPS per kg body weight given to sows at gd 112 elicited a cortisol response without affecting the piglet viability (Supplementary Fig. S1). Therefore, on gd 112, eight sows per dietary treatment were immune stress challenged *i.m.* with 10 µg/kg of LPS dissolved in 2 mL of saline. The remaining eight sows per dietary treatment received a 2 mL injection of saline as a control. Blood samples were collected before LPS administration and 2 h following LPS administration to measure cortisol and cytokine concentrations. Rectal temperature was recorded hourly up to 6 h post LPS injection.

2.2. Offspring treatment and procedures

Within 24 h of birth, litters were standardized to 12 piglets via cross-fostering within treatments; pigs were weaned at 21 days of age. At parturition, each litter was assigned an ID, and each piglet was given a unique ID corresponding to the litter ID. At weaning, pigs (N = 475) were randomly mixed and sorted into 8 pens (n = 15 pigs per pen for the first week after weaning and n = 9 pigs per pen for the remainder of the trial), as is the standard management practice, and were placed on either a high-quality or low-quality protein diet such that half of the pigs from each litter were fed each experimental nursery diet. Piglets were given ad libitum access to feed and had unlimited access to fresh water for the duration of the trial. Nursery diets were formulated to meet the estimated nutrient requirements for nursery phases 1 through 3 (National Research Council, 2012; Table 3) and were formulated to be isonitrogenous and isocaloric. Piglets were fed their respective nursery experimental diets for nursery phases 1 and 2 and were then placed on a common nursery diet for phase 3. Piglets were weighed weekly to monitor growth and calculate average daily gain (ADG) per pen. The addition and disappearance of feed were recorded to assess average daily feed intake (ADFI) and gain-to-feed (G:F) ratio.

2.3. Offspring cytokine analysis following LPS immune stress challenge

On d7 post-weaning, four pigs per litter (n = 2 per nursery dietary treatment, N = 192) were subjected to a 40 µg/kg BW *i.m.* LPS endotoxin immune stress challenge. The LPS challenge was conducted as described by You et al. (2019). Blood samples were collected from animals before LPS challenge and 2 h post-LPS injection. Blood samples were left to clot for 1 h at room temperature (RT) before being centrifuged at 1000 × *g* for 20 min. Serum was aliquoted into 2 mL vials and stored at −80 °C until cytokine analysis could be performed.

A panel of serum cytokines (IL-1β, IL-1ra, IL-6 and IL-10) was analysed using a multiplex assay (Milliplex Map Porcine cytokine/chemokine magnetic bead panel, Millipore, Canada). Briefly, 25 µL of each sample was added to a 96-well plate with 25 µL of antibody-conjugated beads. Plates were sealed, wrapped in aluminum foil, and incubated on a plate shaker for 24 h at 4 °C. After 3 washes with wash buffer, a mixture of anti-porcine IL-1β, IL-1ra IL-6 and IL-10 detection antibodies were added at a total volume of 50 µL/well. The plates were sealed, wrapped in foil, and incubated for 2 h at RT. Following incubation, plates were decanted, 50 µL of Streptavidin-Phycoerythrin

Table 2
Ingredient composition of experimental sow diets.

	3.12% AL[1]	3.1% FO[2]	CO[3]
Days on feed	39	39	39
Ingredient composition (%)			
Corn (NRC[4]; 8.3% CP[5])	74.28	74.06	77.06
Corn Oil	1.55	1.79	1.89
Fish Oil[6]	–	3.10	–
Algae[7]	3.12	–	–
Soybean Meal	17.38	17.38	17.38
L-Lysine	0.07	0.07	0.07
Salt	0.40	0.40	0.40
Limestone	1.52	1.52	1.52
Mono-Cal Phosphate	1.17	1.17	1.17
Vitamin/Mineral Premix[8]	0.50	0.50	0.50
Vitamin E[6]	0.02	0.02	0.02
Calculated Nutrient content[9]			
ME, kcal/kg	3097	3153	3090
CP (%)	14.48	14.61	14.46
SID[10] Lysine (%)	0.64	0.65	0.64
SID Methionine + Cysteine (%)	0.43	0.44	0.43
SID Threonine (%)	0.45	0.46	0.45
SID Tryptophan (%)	0.13	0.13	0.13
Analyzed nutrient content			
Dry matter (%)	86.98	88.17	87.53
CP (%)	14.34	15.03	14.44
Phosphorus (%)	0.57	0.58	0.54
Sodium (%)	0.18	0.17	0.17
Calcium (%)	0.81	0.78	0.88
Potassium (%)	0.65	0.67	0.65
Magnesium (%)	0.14	0.14	0.13
Eicosapentanoic acid (EPA; mg/g)	0.19	3.49	0.08
Docosahexanoic acid (DHA; mg/g)	3.76	3.16	0.03
Total omega-3 PUFA[11] (mg/g)	5.43	10.21	1.70
Ratio n-3:n-6[12]	0.21	0.37	0.07

[1] AL, microalgae.
[2] FO, fish oil.
[3] CO, 1.89% corn oil control.
[4] NRC, National Research Council.
[5] CP, crude protein.
[6] Fish oil and Vitamin E provided by Grand Valley Fortifiers (Cambridge, ON, CA).
[7] Algae provided by Alltech Inc. (Nicholasville, KY, USA) and supplied as dried biomass containing 15.8% CP, 70% CF and 17% DHA.
[8] Supplied per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1200 IU as cholecalciferol; vitamin E, 48 IU as DL-α-tocopherol acetate; vitamin K, 3 mg as menadione; vitamin B12, 0.03 mg; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; biotin, 200 µg; Cu, 18 mg as CuSO4·5H2O; Fe, 120 mg as FeSO4; Mn, 24 mg as MnSO4; Zn, 126 mg as ZnO; Se, 0.36 mg as FeSeO3 ; I, 0.6 mg as KI;DSM.
[9] Calculated on the basis of the NRC (2012) ingredient values.
[10] SID, standardized ileal digestible.
[11] PUFA, polyunsaturated fatty acids.
[12] Ratio n-3:n-6, ratio of omega-3 PUFA to omega-6 PUFA.

Table 3
Ingredient composition of experimental nursery diets.

Diet	Low quality		High quality		Common
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 3
Days on feed	7	14	7	14	21
Ingredient composition (%)					
Corn (NRC[1]; 8.3% CP[2])	45.28	41.01	32.57	46.11	48.8
Wheat	15.00	15.00	15.00	15.00	15.00
Fat, Animal/vegetable blend	5.00	5.00	5.00	5.00	2.50
Soybean meal	30.00	35.00	10.00	15.00	30.00
Soybean protein isolate	–	–	9.30	3.00	–
Whey	–	–	20.00	8.00	–
Blood plasma	–	–	4.50	2.00	–
Blood meal	–	–	–	2.00	–
L-Lysine	0.37	0.26	–	0.30	0.35
L-Methionine	0.15	0.11	0.13	0.14	0.09
L-Tryptophan	0.02	–	–	–	–
L-Threonine	0.24	0.12	0.05	0.15	0.10
Salt	0.50	0.40	–	–	0.40
Limestone	1.30	1.25	0.89	1.11	1.17
Monocalcium Phosphate	1.52	1.24	1.94	1.57	0.97
Mineral/Vitamin premix[3]	0.60	0.60	0.60	0.60	0.60
Vitamin E[4]	0.02	0.02	0.02	0.02	0.02
Calculated nutrient content[5]					
ME, kcal/kg	3471	3478	3552	3536	3367
CP (%)	20.85	22.65	23.40	20.40	20.96
SID[6] Lysine (%)	1.21	1.25	1.25	1.25	1.20
SID Met + Cys (%)	1.20	1.35	0.79	0.71	1.21
SID Threonine (%)	0.48	0.53	0.86	0.81	0.49
SID Tryptophan (%)	0.74	0.82	0.29	0.23	0.75
Analyzed nutrient content (%)					
Dry matter	87.08	87.39	88.05	87.19	86.33
CP	21.18	22.44	20.41	18.97	16.86
Phosphorus	0.63	0.64	0.74	0.66	0.56
Calcium	0.87	0.70	0.85	1.04	1.19
Sodium	0.18	0.18	0.31	0.17	0.17
Potassium	0.89	0.97	0.81	0.68	0.76
Magnesium	0.17	0.17	0.14	0.13	0.15

[1] NRC, National Research Council.
[2] CP, crude protein.
[3] Supplied per kg of diet: vitamin A, 12,000 IU as retinyl acetate; vitamin D3, 1200 IU as cholecalciferol; vitamin E, 48 IU as DL-α-tocopherol acetate; vitamin K, 3 mg as menadione; vitamin B12, 0.03 mg; pantothenic acid, 18 mg; riboflavin, 6 mg; choline, 600 mg; folic acid, 2.4 mg; niacin, 30 mg; thiamine, 18 mg; pyridoxine, 1.8 mg; biotin, 200 µg; Cu, 18 mg as CuSO4·5H2O; Fe, 120 mg as FeSO4; Mn, 24 mg as MnSO4; Zn, 126 mg as ZnO; Se, 0.36 mg as FeSeO3 ; I, 0.6 mg as KI;DSM.
[4] Vitamin E provided by Grand Valley Fortifiers (Cambridge, ON, CA).
[5] Calculated on the basis of the NRC (2012) ingredient values.
[6] SID, standardized ileal digestible.

was added to each well, and plates were incubated for 30 min then washed three times. Finally, 100 µL/well of Luminex® sheath fluid was added, and beads were resuspended on a shaker for 5 min before reading on a Luminex® 200 analyzer (Luminex Corp, USA). Cytokine concentrations were determined by comparing samples to standards of known concentrations provided by the manufacturer. The inter-assay CV was 11.2%.

2.4. Offspring antigen-specific dermal hypersensitivity response (DHR)

Four piglets from each litter (n = 2 per piglet dietary treatment; 1 female and 1 castrated male per nursery diet, N = 192 in total) were antigen sensitized *i.m.* on postpartum d28 with a vaccine containing 0.5 mg/mL ovalbumin (OVA; Sigma-Aldrich, Oakville, Ontario) and 0.5 mg/mL *Candida albicans* cellular antigen (CAA; Greer Laboratories Inc., Lenoir, North Carolina) dissolved in 1 mL of saline containing 0.5 mg/mL of Quil-A adjuvant (Sigma-Aldrich, Oakville Ontario). On postpartum d42, piglets received a booster vaccine with the same concentrations of OVA, CAA and adjuvant. On postpartum d55, piglets were immune challenged *s.c.* with 0.1 mL of OVA or CAA at a concentration of 1 mg/mL on each of the inner thighs, with a saline site as a control on each leg. Skin-fold thickness measurements were collected in triplicate using Harpenden Skin-fold Calipers (Creative Health Products, Ann Arbor, Michigan) before *s.c.* injection, and 3, 6, 24 and 48 h post-antigen challenge. The timeline of events is illustrated in Fig. 1.

2.5. Offspring antigen-specific IgG1 and IgG2 response

Blood was collected from the vaccinated piglets described above on postpartum d28, d42 and d55 in 10 mL serum collection tubes (BD Vacutainer, USA). Samples were left to clot for 1 h at RT, then centrifuged at 1000 × *g* for 20 min. Serum was aliquoted into 2 mL vials and stored at −80 °C until antigen-specific IgG1 and IgG2 analyses were carried out by enzyme linked immunosorbent assay (ELISA).

Antigen-specific IgG1 and IgG2 ELISAs were conducted as per Lee et al. (2019). Briefly, 96-well plates were coated with 1.4 µg/mL of either OVA or CAA, and incubated at 4 °C for 48 h. Following antigen coating, plates were washed 5 times with 0.05% PBS-Tween 20 wash buffer. Plates were incubated at RT with 200 µL/well of blocking solution (Bio-Rad Laboratories, Mississauga, Ontario) for 1 h, washed 5 times with wash buffer, and then 100 µL/well of diluted serum samples (1:800 for IgG1 and 1:50 for IgG2 analyses) were incubated in duplicate for 2 h. After washing, 100 µL/well of primary mouse anti-pig IgG1 or IgG2 antibodies (Bio-Rad Laboratories, Mississauga, Ontario) were added to each well and incubated for 1 h at RT, then washed again and incubated with conjugated goat anti-mouse IgG (Sigma-Aldrich, Oakville, Ontario) for 1 h. After a final wash, 80 µL/well of alkaline phosphatase yellow substrate (Sigma-Aldrich, Oakville, ON) was added to each well and incubated for 30 min at RT in the dark. A standard curve, as well as positive and negative control samples, were added to each plate. Following the 30 min substrate incubation, plates were analyzed at 405 nm using a plate reader (Victor[3] 1420 Multilabel Counter, Perkin Elmer, USA); the inter-assay CV was 12.9%.

2.6. Offspring antigen-specific histamine analysis

Whole blood was collected from the pigs described above that received OVA and CAA vaccines on d56 post-weaning in 10 mL heparinized collection tubes for histamine analysis. Briefly, 1 mL of blood was placed in 24-well plates along with 1 mL of 10 µg/mL OVA dissolved in saline. Blood supernatant, which was collected by spinning plates at 1000 x g for 15 min, was collected immediately before the addition of OVA antigen as well as after a 30 min incubation at 37 °C. Supernatants were kept at −80 °C until histamine analysis was conducted.

Histamine analysis was conducted using a commercial assay (Histamine ELISA kit, Fitzgerald Industries International). Briefly, 25 µL of sample supernatant was combined with 25 µL of acylation buffer and 25 µL of acylation solution in a 2 mL vial, and vials were incubated at RT for 45 min on a shaker. Samples were then diluted with 100 µL of water before incubation on a shaker at RT for another 15 min. Samples were then diluted 1:8 with water, and 25 µL of diluted sample was placed in each well of a 96-well plate, along with 100 µL of histamine antibodies. The plate was then covered with adhesive foil and incubated on a shaker for 3 h at RT. Plates were then washed 4 times with wash buffer (PBS-tween) before adding 100 µL/well of enzyme conjugate, which was then incubated for 30 min on a shaker. Plates were again washed 4 times with wash buffer before adding 100 µL/well of substrate and incubating for 20 min in the dark on a shaker. Finally, 100 µL/well of stop solution was added to each well and absorbance was read at 450 nm using a plate reader (Victor[3] 1420 Multilabel Counter, Perkin Elmer, USA); the inter-assay CV was 0.6%.

2.7. Statistical analysis

Statistical analysis was conducted using PROC GLIMMIX of SAS version 9.4. A multiple means comparison was conducted for the data from the preliminary LPS trial with sow as the experimental unit and LPS dose as the fixed effect. A preliminary PUFA analysis was conducted using a multiple means analysis with sow as the experimental unit and gestation dietary treatment as the fixed effect. A repeated measures analysis was used for the piglet performance data, DHR and antigen-specific IgG1 and IgG2 responses to compare data across timepoints, and multiple means comparisons were carried out for the cytokine and histamine data; treatment least squared means (LSM) were obtained for all variables. The statistical model for the nursery performance data used pen as the experimental unit and the fixed effects of nursery dietary treatment (high- or low-quality protein), time, and their interactions; sow and block were included as random effects. The statistical model for the DHR response, antigen-specific IgG1 and IgG2 response, histamine analysis and cytokine analysis used pig as the experimental unit and included the fixed effects of gestation dietary treatment (microalgae, fish oil, corn oil control), maternal LPS status (LPS or CON) and nursery dietary treatment (high- or low-quality), time (i.e. day post weaning), and their interactions; block and sow were included as random effects. Where fixed effects were not significant ($P > 0.05$), a reduced model was used. Significant differences were reported at $P < 0.05$, and trends were reported between $0.05 \leq P \leq 0.1$.

3. Results

3.1. Sow feeding trial and LPS immune stress challenge

Rectal temperature and cortisol response in sows following a 10 μg/kg LPS immune challenge were presented in You et al. (2019) and are shown in Supplementary Fig. S2. A significant effect of LPS status was observed; sows challenged with 10 μg/kg of LPS had increased rectal temperature compared to those receiving saline (CON) 2 h post LPS immune stress challenge and remained significantly elevated between 2 and 6 h post-LPS immune stress challenge ($P < 0.05$), and was significantly decreased in sows fed the fish oil diet compared to those fed microalgae or corn oil control diets at 4, 5, and 6 h post LPS immune stress challenge ($P < 0.05$). Serum cortisol analysis indicated that sows challenged with 10 μg/kg LPS had elevated serum cortisol concentrations 2 h post LPS immune stress challenge compared to those receiving saline (CON). Sow reproductive traits were also assessed and included gestation length, litter size, number of stillborn piglets, litter weight at birth and number of piglets weaned, and no significant differences between gestation dietary treatments or maternal LPS status were observed.

3.2. Offspring performance

During the lactation phase, before weaning, piglet ADG was not different among maternal gestation dietary treatments or between maternal LPS status. Due to the use of pen as the experimental unit after weaning, comparisons in performance could only be made between nursery dietary treatment groups. There was a significant interaction of phase of feeding and high- or low-quality protein piglet diets for ADG, ADFI and G:F ($P < 0.01$; Table 4). The ADG ($P < 0.0001$), ADFI ($P < 0.0001$) and G:F ($P < 0.01$) were increased in offspring fed the high-quality protein nursery diet in phase 1, but were not different between offspring nursery dietary treatments for phases 2 or 3, or overall.

3.3. Offspring cytokine analysis following LPS immune stress challenge

Results from multiplex cytokine analysis showed a significant time effect, with greater serum concentrations of IL-1β, IL-1ra, IL-6 and IL-10 2 h after LPS immune challenge ($P < 0.05$; Fig. 2). A significant interaction was found between offspring nursery dietary treatment and time of sampling for IL-1ra, IL-6 and IL-10 concentrations, with greater cytokine concentrations in pigs fed a high-quality protein diet compared to pigs fed a low-quality protein diet 2 h after LPS immune challenge ($P < 0.05$). No significant effects of gestation dietary treatment or maternal LPS status were observed for serum concentrations of IL-1β, IL-1ra, IL-6 and IL-10, and no effect of nursery dietary treatment was observed for concentrations of IL-1β.

Table 4
Body weights, average daily gain (ADG), average daily feed intake (ADFI) and gain: feed ratio (G:F) for pigs from weaning to end of Phase 3 fed diets with either high- (n = 240) or low-quality (n = 240) protein sources. Results presented as LSM +/- SEM.

Diet	High quality	Low quality	SEM[1]	P-value[2]
Body weight, kg				
Weaning	6.80	7.41	0.28	0.13
D7	7.72	8.01	0.28	0.46
D21	12.09	13.07	0.46	0.14
D42	25.12	26.77	0.76	0.14
ADG[3], g				
Phase 1	**132**	**84**	**14.71**	**< 0.0001**
Phase 2	361	378	23.61	0.60
Phase 3	608	642	17.87	0.12
Overall	404	418	13.70	0.41
ADFI[4], g				
Phase 1	**210**	**181**	**17.91**	**< 0.0001**
Phase 2	474	486	43.37	0.55
Phase 3	1066	1097	44.24	0.47
Overall	665	666	35.73	0.94
G:F[5]				
Phase 1	**0.64**	**0.45**	**0.047**	**0.0003**
Phase 2	0.76	0.79	0.054	0.59
Phase 3	0.57	0.59	0.011	0.26
Overall	0.61	0.63	0.016	0.25

[1] SEM, standard error of means.
[2] P-value for main effect of dietary treatment.
[3] ADG, average daily gain.
[4] ADFI, average daily feed intake.
[5] G:F, gain to feed ratio.

3.4. Offspring antigen-specific dermal hypersensitivity response

Results from the dermal hypersensitivity challenge showed changes in skin-fold thickness in response to both OVA and CAA antigens over time ($P < 0.0001$; Supplementary Fig. S3), indicating that the antigen challenge was successful in eliciting an immune response. In response to OVA antigen, skin-fold thickness was greatest at the 3 h timepoint, and subsequently decreased over time. In response to CAA, skin-fold thickness increased at the 3-, 6- and 24-h time points, and was significantly lower by the 48-h time point. Maternal gestation dietary treatment, maternal LPS status, or offspring nursery dietary treatment did not influence offspring DHR at any time point for either antigen.

3.5. Offspring piglet antigen-specific antibody response

OVA-specific serum IgG1 was influenced by the main effect of time ($P < 0.0001$; Supplementary Fig. S4); serum IgG1 concentration was greater at 28 d versus baseline and 14 d after initial vaccination, which indicates that the animals responded to the vaccination protocol. Gestation dietary treatment, maternal LPS challenge, and nursery dietary treatment did not influence offspring OVA-specific serum IgG1.

For CAA-specific serum IgG2 concentration, there was a significant interaction of gestation dietary treatment, offspring nursery dietary treatment, and time; only offspring fed the high-quality protein diet from sows fed the microalgae diet and pigs fed the low-quality diet from

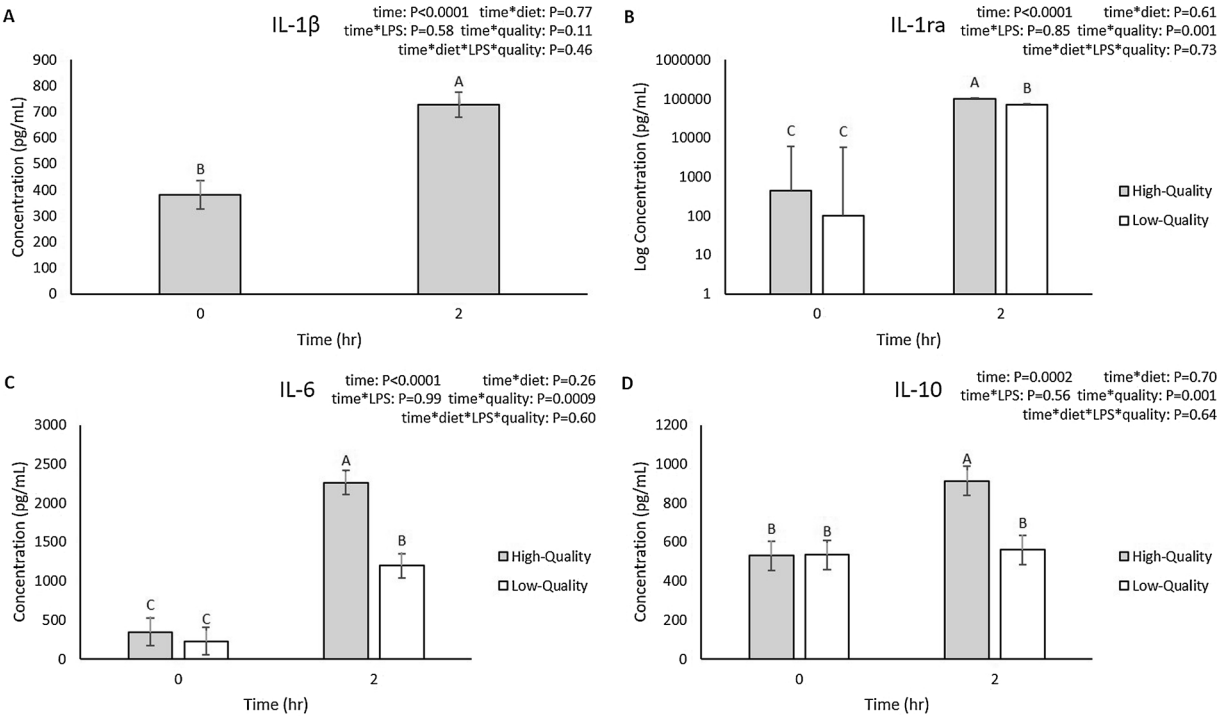

Fig. 2. Cytokine levels in offspring piglets following a 40 μg/kg *i.m.* lipopolysaccharide (LPS) immune stress challenge (n = 188): (A) IL-1β, (B) IL-1ra, (C) IL-6 and (D) IL-10 concentrations in piglets fed either a high- or low-quality protein diet (n = 94 per treatment). Results are presented as LSM +/− SEM. Significant differences ($P < 0.05$) between treatments are denoted with differing letters above bars. P-values for analysis of fixed effects in the statistical model are shown in the upper right-hand corner of the figure. **Time**: time of sampling, **diet**: maternal AL, FO or CO diet, **LPS**: maternal LPS status, **quality**: high- or low-quality piglet diet.

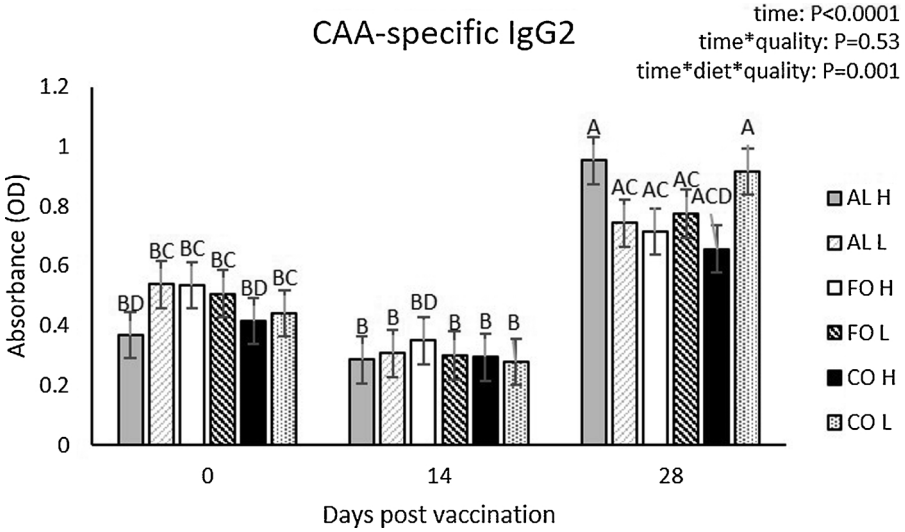

Fig. 3. *Candida albicans* (CAA)-specific antibody levels in piglets fed either a high- or low-quality protein diet from dams fed diets supplemented with 3.12% microalgae (AL), 3.1% fish oil (FO) or 1.89% corn oil (CO) as a control and challenged with 10 μg/kg LPS or (n = 190). Results are presented as LSM +/− SEM. Significant differences ($P < 0.05$) are denoted with differing letters above bars. P-values for analysis of fixed effects in the statistical model are shown in the upper right-hand corner of the figure. **Time**: day of sampling, **diet**: maternal AL, FO or CO diet, **LPS**: maternal LPS status, **quality**: high- or low-quality piglet diets.

sows fed the corn oil control diet had relatively greater CAA-specific IgG2 concentrations 28 d after initial vaccination in compared to their respective basal and 14 d CAA-specific IgG2 concentrations ($P < 0.0001$; Fig. 3). Overall, CAA-specific IgG2 concentrations increased 28 d after initial vaccination compared to baseline sampling ($P < 0.0001$) and to 14 d after initial vaccination ($P < 0.0001$). Maternal gestation dietary treatment, maternal LPS status, and offspring nursery dietary treatment did not influence offspring CAA-specific IgG2 response within each sampling time point.

3.6. Offspring piglet antigen-specific histamine analysis

Histamine response following whole blood stimulation with OVA antigen showed a significant time effect, with an increase in histamine release 30 min after OVA stimulation ($P < 0.0001$; Supplementary Fig. S5). Maternal gestation dietary treatment, maternal LPS status and offspring nursery dietary treatment did not influence offspring histamine response.

4. Discussion

This study aimed to investigate the effects of transitioning piglets from differing maternal treatments onto nursery diets containing high- or low-quality protein sources. Previously, feeding a low-quality protein diet had no effect on the fever response following an *i.m.* LPS immune stress challenge (Dritz et al., 1996; Huber et al., 2018), and had no effect on concentrations of the pro-inflammatory cytokines TNF-α and IFN-γ (Paßlack et al., 2017). In the present study, significant increases

in the concentrations of the cytokines IL-1ra, IL-6 and IL-10 were observed 2 h after LPS immune stress challenge in pigs fed the high-quality protein diet compared to those fed the low-quality protein diet. While IL-6 can have either pro-inflammatory or anti-inflammatory effects that are dependent on its receptor (Hunter and Jones, 2015), IL-1ra and IL-10 are considered anti-inflammatory (Arend, 2002; Gabryšová et al., 2014; Hunter and Jones, 2015). Due to the anti-inflammatory nature of IL-10 and IL-1ra, their decreased production in piglets fed a low-quality protein diet compared to their high-quality counterparts may leave these animals vulnerable to future innate immune stimulation and inflammatory disease. In this study, the production of IL-10 and IL-1ra are likely induced to counteract the effects of IL-1β and IL-6, and it is possible that the time of sampling may not represent the peak or overall activity of the measured cytokines, as Granowitz et al. (1991) showed different peak times and peak concentrations for IL-1ra and IL-1β. Despite the effects of piglet diet quality on cytokine response, You et al. (2019) showed no effect of diet quality on the fever and cortisol responses of these same piglets. Cytokine production typically drives the fever response, and some of the most important cytokines involved in eliciting a fever response include TNF-α and IL-1β (Poon et al., 2015). In the present study, IL-1β concentrations were not affected by offspring nursery dietary treatment, and in previous research, TNF-α concentrations were also unaffected by dietary protein quality (Paßlack et al., 2017). The lack of differences in concentrations of these two pyrogenic cytokines likely explains why the fever response to LPS between pigs fed a high- or low-quality protein diet was not significantly different.

A study by Huber et al. (2018) found no differences in the dermal hypersensitivity response to either OVA antigen, or in the OVA-specific IgG response in pigs fed a low-quality protein diet compared to those fed a high-quality protein diet; this was also observed in the present study. The results obtained from the acquired immune challenge, in addition to the results of the cytokine analysis, suggest that in swine, the acute-phase response may be more sensitive to changes in dietary protein quality compared to the adaptive immune response.

This is one of the first studies using microalgae as a dietary source of n-3 PUFA in late gestation sows in addition to using the LPS endotoxin stress model to simulate bacterial infection and to investigate the effects of maternal treatments on piglet growth and health after weaning. Both maternal and fetal tissue enrichment with n-3 PUFA was shown to occur following dietary supplementation with fish oil and microalgae. You et al. (2019) demonstrated that fish oil supplementation of these same sows reduced their fever response to LPS immune stress challenge, whereas microalgae supplementation had no effect on rectal temperature. You et al. (2019) also demonstrated that the LPS immune challenge elicited a cortisol response in these same sows, but this response was unaffected by maternal diet.

It was anticipated that maternal dietary supplementation with fish oil or microalgae would help protect offspring from post-weaning LPS-induced stress. You et al. (2019) showed that when these same offspring were LPS immune challenged, their cortisol response was influenced by maternal gestation dietary treatment and maternal LPS status. However, our analysis of cytokine biomarkers in these piglets revealed no effects of maternal treatment. We have previously observed that including microalgae in the diets of weanling pigs increased the production of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and the anti-inflammatory cytokine IL-10 during LPS immune challenge in comparison to pigs fed diets supplemented with fish oil or corn oil (Lee et al., 2019). This suggests that cortisol and fever responses may occur independently of a systemic cytokine response. Previously, Upadhaya et al. (2015) also found no differences in the inflammatory cytokine response following an *i.m.* LPS immune challenge in growing-finishing pigs. The acute-phase response to LPS immune stress challenge may be influenced by the route of LPS administration, which in this study was performed *i.m.*; it has been previously shown that route of LPS administration differentially affected the fever response in rabbits and in guinea pigs (Cartmell et al., 2002; Goldbach et al., 1997) and differentially affected cytokine and acute-phase protein concentrations in pigs (Wyns et al., 2015).

Maternal supplementation with n-3 PUFA can modulate the offspring acquired immune response assessed in vitro, in humans and in sheep (Fisher-Heffernan et al., 2015; Miles and Calder, 2017; Quin et al., 2016). However, no biologically relevant effects of maternal gestation dietary treatment, maternal LPS status or offspring nursery dietary treatment were observed in the present study for skin-fold thickness following dermal hypersensitivity challenge or OVA- and CAA-specific antibody analyses. These results contrast with previous research, where an increase in IgG antibodies was observed in pigs fed a diet high in n-3 PUFA from sows that were also fed a high n-3 PUFA diet (Bazinet et al., 2004). The present results are also in contrast to studies where maternal neurogenic and psychogenic stress, including LPS immune stress and maternal restraint, reduced levels of total IgG in swine and rat offspring (Tuchscherer et al., 2002; Veru et al., 2014), and reduced CAA-specific IgG in sheep offspring (Fisher-Heffernan et al., 2015). These differences may be partially attributed to species differences, or to the dose and route of LPS administration. As route of administration and dose of LPS differentially affect the acute-phase response in rabbits and pigs (Cartmell et al., 2002; Wyns et al., 2015), they may also differentially modulate offspring cell-mediated and antibody-mediated immunity. The timing of the maternal stressor may also be an important factor in the observed results; the timing of a maternal stressor can greatly influence the outcomes and effects on the offspring (Veru et al., 2014; Vieau et al., 2007). In the present study, the maternal LPS stress challenge occurred on gd112 and parturition on gd114; though the results from this LPS challenge are indicative of a stress response (You et al., 2019), more pronounced effects on the offspring acquired immunity may have been observed had the LPS stress challenge occurred at an earlier point during gestation. The day of gestation selected for the sow LPS challenge was based on a preliminary study conducted by our group; administration of LPS at an earlier gestation date resulted in substantial piglet mortality and decreased piglet viability.

Due to difficulties in animal management after weaning, the way in which the pigs were housed could not account for the effects of maternal diet or maternal LPS status on post-weaning piglet growth. Limited effects of maternal n-3 PUFA supplementation have been observed on piglet growth; a meta-analysis by Tanghe and De Smet (2013) showed a slight effect of maternal diet on piglet birth weight and growth during lactation, but showed no effect of maternal diet on growth in the post-weaning phase. Similarly, no differences have been observed in the growth of lambs, regardless of maternal LPS status or maternal fishmeal or soybean meal supplementation (Fisher-Heffernan et al., 2015; Fisher et al., 2014). However, future studies should be designed in such a manner as to account for maternal diet and maternal LPS status to assess the effects of these treatments on piglet performance, perhaps from weaning until the end of the production cycle, when the pigs reach final market weight.

5. Conclusions

Overall, maternal dietary supplementation with microalgae or fish oil with or without a maternal LPS immune challenge in late gestation had no apparent effect on piglet acquired immune response from weaning to 6 weeks post-weaning in this trial. Protein quality of piglet diets impacted acute-phase response as indicated by cytokine concentrations on d28 and growth of pigs only during the first phase after weaning. While pigs fed the high-quality protein diet had better ADG and feed efficiency in nursery phase 1, this effect disappeared in phases 2 and 3 and resulted in similar growth and feed intake over the entire nursery period. Cytokine production was affected by nursery diet, with pigs fed the high-quality protein diet having greater serum concentrations of IL-1ra, IL-6 and IL-10 2 h post-LPS immune stress challenge. In

this instance, piglet diet appears more important for predicting growth and acute-phase response to microbial stressors than maternal diet or maternal immune system stimulation during late gestation. These results also suggest that feeding a low-quality protein diet in the nursery phase may leave piglets vulnerable to inflammatory disease due to decreased production of anti-inflammatory cytokines, but further validation that includes pathogen challenge will be required to test this hypothesis.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109937.

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