RESEARCHREPORT

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PORK SAFETY

Title: Immunomodulatory and Growth Effects of the Seaweed *Ascophyllum nodosum* in Pigs – **NPB #00-063**

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I. Abstract:

A series of *in vitro* studies was conducted to evaluate the effect of *A. nodosum* extract (**ANE**) on activation of pig splenocytes and alveolar macrophages. In the first set of studies, splenocytes and macrophages were incubated with concentrations of **ANE** from .1-100 µg/mL. Concentrations of TNFα and PGE_2 were evaluated as measures of macrophage activation and interleukin 10 (IL-10) and interferon gamma (INFγ) were evaluated as measures of splenic lymphocyte activation. As positive controls, macrophages were stimulated with bacterial lipopolysaccharide (LPS) and splenocytes were incubated with the mitogen concanavalin A (Con A). **ANE** failed to stimulate secretion of PGE_2 or any cytokine by cultured immune cells. In a subsequent set of experiments, concentrations of **ANE** as high as 10 mg/mL were evaluated. Again, **ANE** did not alter IL-10 production by splenocytes. However, PGE_2 was increased ($P < .05$) relative to control wells in macrophages treated with 10 mg/mL **ANE** after 3 and 24 hours in culture. The results of our *in vitro* studies demonstrate conclusively that **ANE**, when presented directly to immune cells across a wide range of concentrations, generally failed to activate macrophages and lymphocytes. These immune cells are key players in both cell-mediated and antibody-mediated immune function. Very high concentrations of **ANE** enhanced production of the inflammatory mediator PGE_2, but never to the degree achieved by bacterial LPS.

An *in vivo* study also was conducted with dietary **ANE**. A total of 95 pigs (initially 15 lb and 17 d of age) was used in a 28 d growth experiment to determine the effects of **ANE** on weanling pig growth performance and immune function in response to enteric disease challenge with *Salmonella typhimurium* (**ST**). Experimental treatments were arranged in a 2 x 4 factorial with main effects of disease challenge (control vs. **ST** challenge) and dietary addition of **ANE** (0, 0.5, 1.0, and 2.0% of diet). Results suggest little beneficial effect of dietary **ANE** on growth performance or immune response in the presence or absence of **ST** challenge.

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II. Introduction:

Antibiotics are used extensively in livestock feeding to prevent infection and to improve growth performance and feed efficiency. Today, the use of antibiotics in animal diets has come under increased public scrutiny due to concern about the development of antibiotic-resistant organisms. Management programs (e.g., segregated early weaning, all-in/all-out production, etc.) help to minimize exposure to pathogens; however, acute disease challenges still occur. Therefore, research studying natural alternatives to dietary antimicrobials is on the rise.

Extracts of certain seaweeds have been shown to have antitumor effects in rodents, inhibiting mammary tumors and lung metastases. In addition, extracts of the marine algae *Porphyra yezoensis* activated murine macrophages, enhancing their proinflammatory cytokine production. In addition, preliminary research at another university suggested that dietary addition of **ANE** might enhance growth performance and alter immune function in virally infected nursery pigs.

Our overall hypothesis was that the dietary inclusion of **ANE** would enhance growth and immune function in disease-challenged pigs and that **ANE** would directly modulate the function of pig immune cells.

III. Objectives:

To test our hypothesis, we proposed to:

1) Evaluate the effects of **ANE** extract on activation of pig alveolar macrophages and splenic lymphocytes.

2) Evaluate the effects of **ANE** supplementation on growth and bacterial shedding in pigs infected with **ST**.

IV. Procedures:

Objective 1:

Samples of spleen were obtained from nursery age pigs. Splenocytes were mechanically dispersed, placed in RPMI media that was supplemented with fetal bovine serum and placed in culture in a humidified 95% air, 5% CO_2 atmosphere at 37° C. Alveolar macrophages were obtained by lung lavage with sterile PBS. Cells were pelleted, placed in sterile RPMI media, and plated into 24 well plates. After 3 h in culture, nonadherent cells were removed and plates were cultured as described for splenocytes. Two experiments were conducted with each cell type (Experiments 1a and 2a with macrophages and Experiments 1b and 2b with splenocytes).

For Experiment 1a, alveolar macrophages were exposed to media containing the following: control (no added test substances), bacterial lipopolysaccharide (LPS, 10 ng/mL as a positive control for activation) and **ANE** at .1, 1, 10 and 100 μg/mL. Culture media was removed at 3, 6, 12, and 24 h of treatment and frozen. Macrophage culture media was assayed for TNFα and PGE_2 using enzyme-linked immunosorbant assays (ELISA) procedures. For Experiment 1b, splenocytes were subjected to the same treatment array as in Experiment 1a except that media was removed after 12, 24, and 48 h. Splenocyte media were assayed for IFNγ and IL-

10, again using specific ELISA techniques.

Experiments 2a (macrophages) and 2b (splenocytes) were conducted similarly to Experiments 1a and 1b, except that increased concentrations of **ANE** were employed (.01, .1, 1, and 10 mg/mL for Experiment 2a and.005, .05, .5, and 5 mg/mL for Experiment 2b). Only PGE_2 (macrophages) and IL-10 (splenocytes) were evaluated as measures of activation.

Data were analyzed within time in culture using the mixed model procedure of SAS. The model included effects of treatment, and pig was treated as a random source of variation in the model.

Objective 2:

The KSU Institutional Animal Care and Use Committee approved the experimental protocol used in this study. A total of 95 pigs (initially 15 lb and 17 d of age) were blocked by initial weight, equalized for sex, and randomly allotted to one of eight treatments in a 28 d growth assay. Each treatment had six replicates (pens) with two pigs per pen.

The eight treatments were arranged in a 2 x 4 factorial with main effects of disease challenge (control or **ST**) and dietary treatment (0, 0.5, 1.0, or 2.0% **ANE**). The **ANE** extract used in this study was obtained from Acadian Seaplants Limited, Nova Scotia, Canada.

All pigs were housed in two similar environmentally controlled rooms according to disease challenge. Pens contained one self-feeder and one nipple waterer to provide *ad libitum* access to feed and water. Prior to the start of the study, fecal samples were taken to ensure that all pigs were free of Salmonella. Pigs were weighed and feed disappearance was measured on d 0, 7, 14, 21, and 28 to determine ADG, ADFI, and F/G. On d 14, each pig housed in the **ST** room (n=48) received approximately 6×10^9 CFU of *S. typhimurium* in 10 ml of growth media. Each pig housed in the control room (n=47) received a similar volume of sterile growth media. Rectal temperature was measured on one pig per pen through 7 d after challenge. Also, daily feed intake was monitored through 7 d after challenge. On d 0, 7, and 14 with respect to challenge, serum samples were obtained from one pig per pen and analyzed for haptoglobin, α1-acid glycoprotein (AGP), IgG and IgM. On d 7 and 14 after challenge, fecal samples were obtained from all pigs and cultured for Salmonella.

Data were analyzed as a 2 x 4 factorial in a randomized complete block design replicated over time using the mixed model procedure of SAS. All means presented are least-square means.

V. Results:

Objective 1:

Experiment 1a. The results are presented in Figure 1. Control macrophages in culture did not produce significant amounts of PGE_2 or TNFα. As expected, LPS

stimulated both PGE_2 and $TNF\alpha$ at all times measured ($P < .05$ compared to control wells and other treatments). Alveolar macrophages did not respond with increased PGE_2 or $TNF\alpha$ to any of the concentrations of **ANE** tested.

Experiment 1b. The results are presented in Figure 2. Control splenocytes in culture did not produce significant amounts of $IFN\gamma$ or IL-10. However, as expected, Con A stimulated both $IFN\gamma$ and IL-10 from splenocytes at all times measured ($P < .05$ compared to control wells and other treatments). In contrast, no concentrations of **ANE** tested affected cytokine production.

Experiment 2a. The results are presented in Figure 3, top panel. Similar to the results from Experiment 1a, macrophages had increased PGE_2 in response to LPS ($P < .05$). In contrast to Experiment 1b, the highest concentration of **ANE**, 10 mg/mL, stimulated PGE_2 production (compared to control), incrementally after 3 h in culture ($P < .05$) and more substantially at 24 h ($P < .001$). However, the increase in PGE_2 was not as great as that obtained by LPS. No other intermediate concentrations of **ANE** affected PGE_2 compared to controls.

Experiment 2b. The results are presented in Figure 3, bottom panel. Similar to the results from Experiment 1b, splenocytes had increased IL-10 in response to Con A ($P < .05$). However, IL-10 secretion was not enhanced with even greater concentrations of **ANE**.

Objective 2:

No differences ($P > .10$) in ADG, ADFI, or F/G occurred between dietary treatments (Table 1). However, a challenge by time interaction ($P < .0005$) was observed. Prior to challenge, ADG, ADFI, and F/G were similar between control and **ST**-challenged pigs. As expected, the **ST** challenge resulted in reductions in ADG ($P < .0001$), ADFI ($P < .005$), and F/G ($P< .002$) compared to controls during wk 3 of the study (Table 1). However, by wk 4, ADFI did not differ ($P>.10$) between control and **ST**-challenged pigs. **ST**-challenged pigs also had improved ADG and F/G compared to controls in wk 4 ($P < .05$).

During the 7 d after bacterial challenge, a challenge by time interaction ($P < .0001$) affected both daily feed intake and rectal temperature. Daily feed intake for **ST**-challenged pigs began to decline ($P < .05$) between 24 to 48 h after challenge (Table 2), but returned to levels comparable to those of controls by 5 d after challenge. Surprisingly, **ST**-challenged pigs had a slightly, but significantly ($P < .05$) increased rectal temperature compared to controls on d 0. Rectal temperatures in **ST**-challenged pigs were elevated on d 1 ($P < .05$), peaked on d 2 ($P < .05$), and returned to control levels by 4 d after challenge.

Dietary **ANE** did not affect serum haptoglobin or α 1-acid glycoprotein (Figure 4), however, there was a significant disease challenge x time interaction for both acute phase proteins ($P < .001$). Unexpectedly, haptoglobin was increased in control pigs before bacterial challenge ($P < .05$). At 7 and 14 d post-challenge, haptoglobin did not differ significantly between control and **ST**-challenged pigs, although concentrations were numerically greater in **ST**-challenged pigs. In

contrast to haptoglobin, AGP was elevated in **ST**-challenged pigs at 7 and 14 d post-infection ($P < .05$).

Neither diet nor disease challenge affected circulating IgG or IgM, but IgM decreased over time ($P < .001$), whereas IgG tended to increase ($P < .07$) over time in all pigs (Figure 4).

Dietary **ANE** did not affect the proportion of pigs shedding Salmonella in feces. As expected, significant numbers of pigs challenged with *S. typhimurium* cultured positive for Salmonella in fecal samples both at 7 and 14 d post-challenge. At 7 d, fecal cultures for control pigs were negative, but 21.3% (10/47; one **ST**-challenged pig died prior to fecal culturing) of challenged pigs were shedding. At 14 d following infectious challenge, 10.6% (5/47) of **ST**-challenged pigs were shedding. At that time, one control pig also was cultured positive for Salmonella. Rectal temperature and serum acute phase protein concentrations for this pig were never elevated, suggesting that the culture for this pig may have been a false positive.

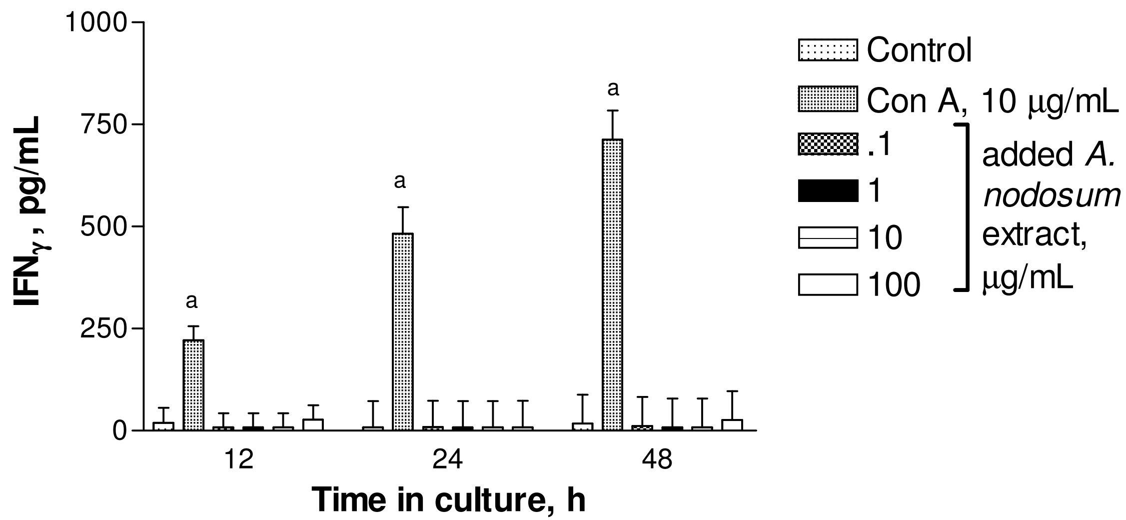
VI. Summary:

The results of our *in vitro* studies demonstrate conclusively that **ANE**, when presented directly to immune cells across a wide range of concentrations, generally failed to activate macrophages and lymphocytes. These immune cells are key players in both cell-mediated and antibody-mediated immune function. Very high concentrations of **ANE** enhanced production of the inflammatory mediator PGE_2, but never to the degree achieved by bacterial LPS.

Regarding our *in vivo* study with weaned pigs, our results suggest little beneficial effect of dietary **ANE** on growth performance or immune response in the presence or absence of **ST** challenge.

Thus, taken together, the data fail to support our original hypothesis and do not provide adequate justification for incorporation of **ANE** in diets for weaned pigs.

Splenocytes



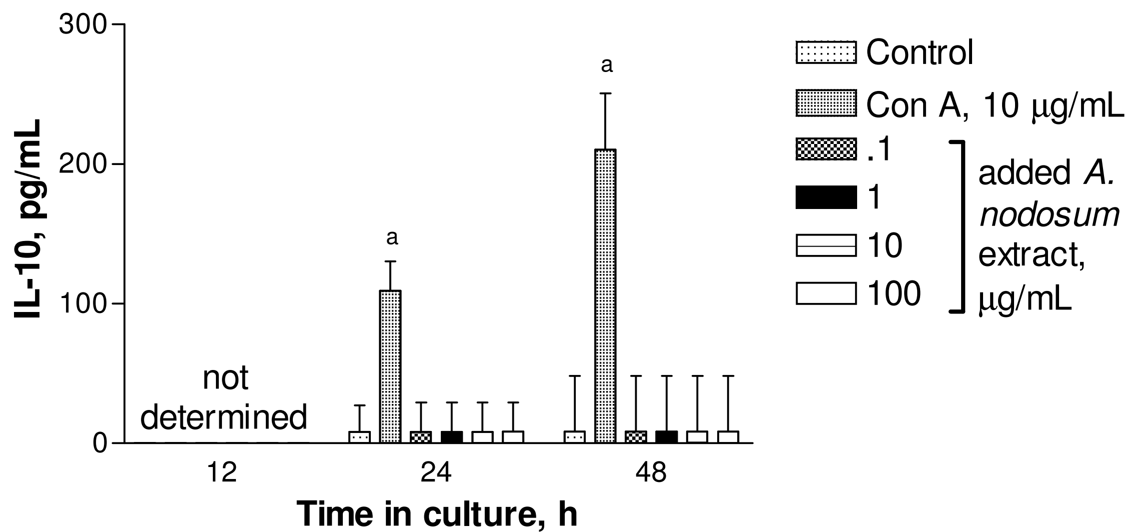
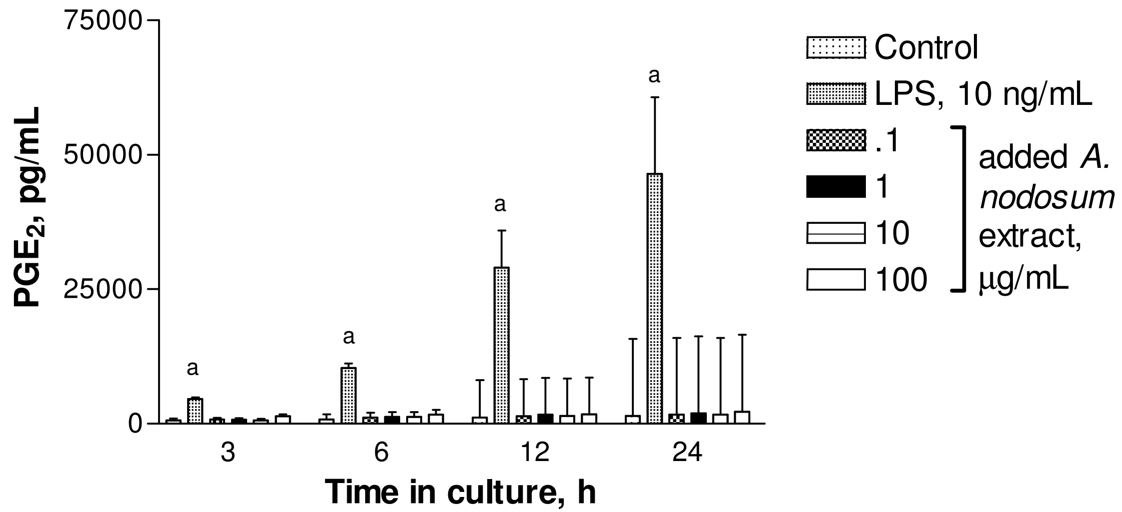


Figure 1. ***In vitro*** **production of prostaglandin E_2 (PGE_2) and tumor necrosis factor alpha (TNFα) in cultured porcine alveolar macrophages in Experiment 1a. Superscripts denote significant ($P < .05$) increases in wells receiving lipopolysaccharide (LPS) compared to control wells and other treatments within a given time in culture.**

Alveolar Macrophages



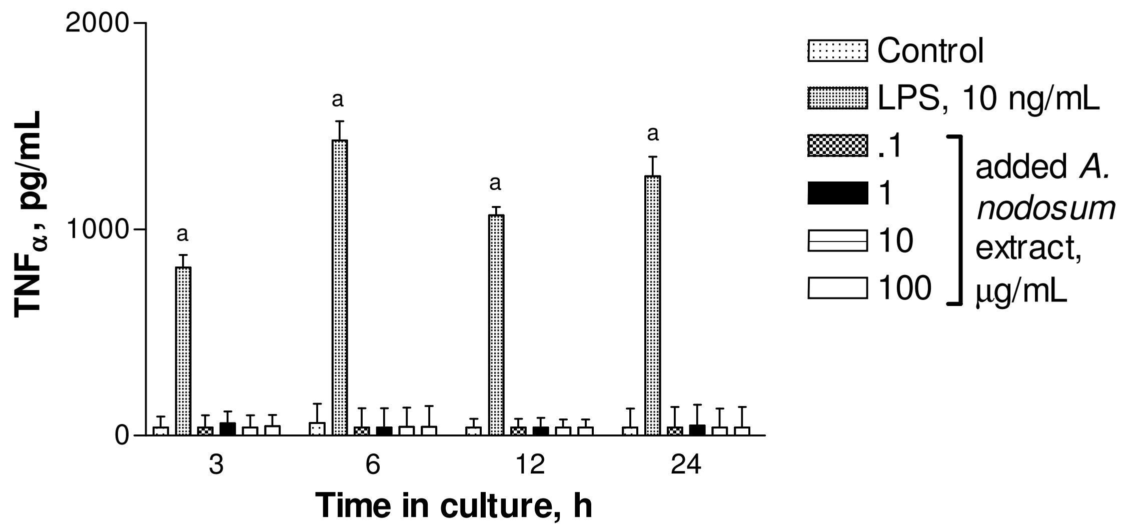
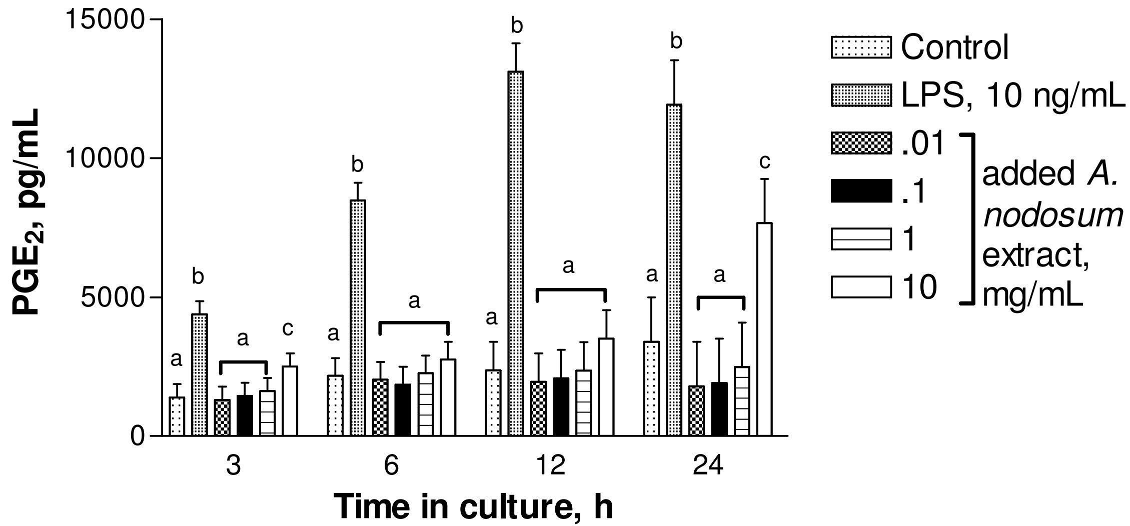


Figure 2. ***In vitro*** **production of interferon gamma (IFNγ) and interleukin 10 (IL-10) in cultured porcine splenocytes in Experiment 1b. Superscripts denote significant ($P < .05$) increases in wells receiving concanavalin A (Con A) compared to control wells and other treatments within a given time in culture.**



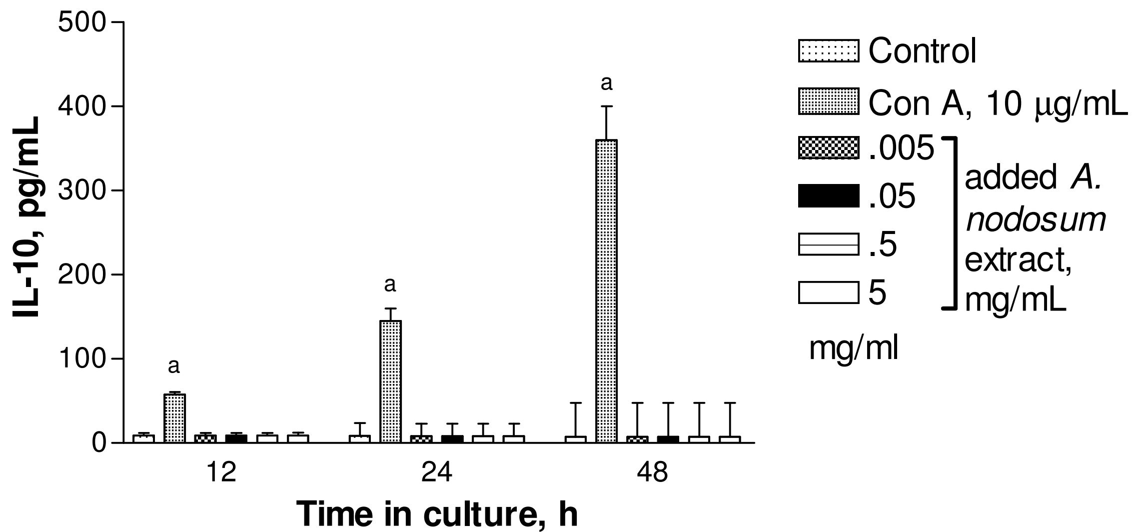
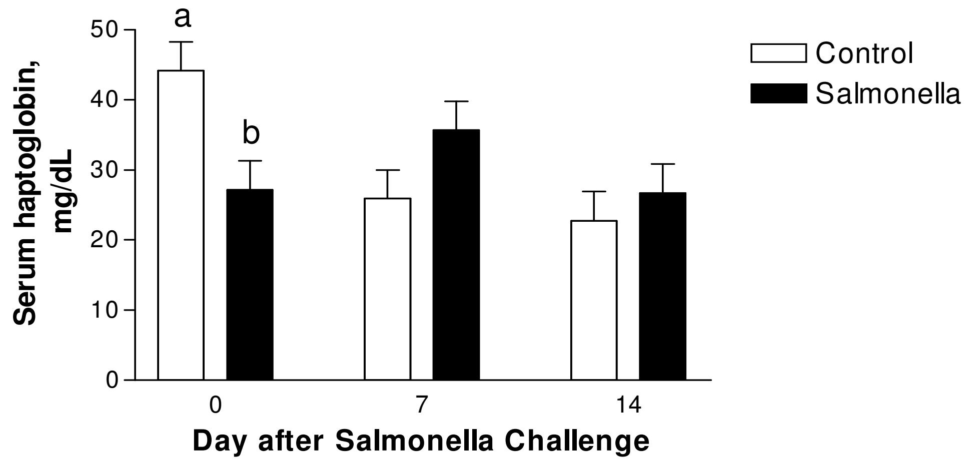


Figure 3. ***In vitro*** **production of prostaglandin E_2 (PGE_2; Experiment 2a) and interleukin 10 (IL-10; Experiment 2b) in cultured pig alveolar macrophages and splenocytes, respectively. For alveolar macrophages within the same time in culture, bars with different superscripts differ statistically ($P < .05$). For splenocytes within the same time in culture, superscripts denote significant increases in Con A-stimulated splenocytes compared to all other treatments ($P < .001$).**



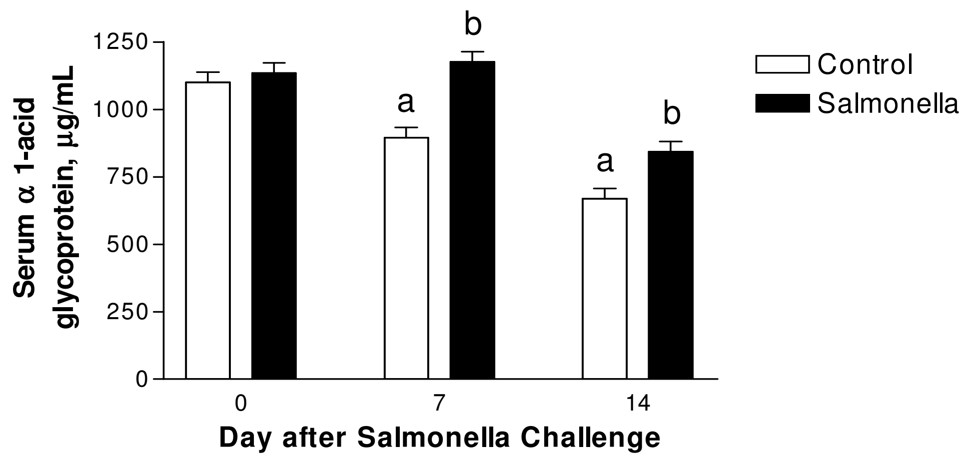


Figure 4. Serum haptoglobin and α1-acid glycoprotein in pigs following Salmonella challenge. Within day following challenge, superscripts denote differences between control and Salmonella-challenged pigs (P < .05).

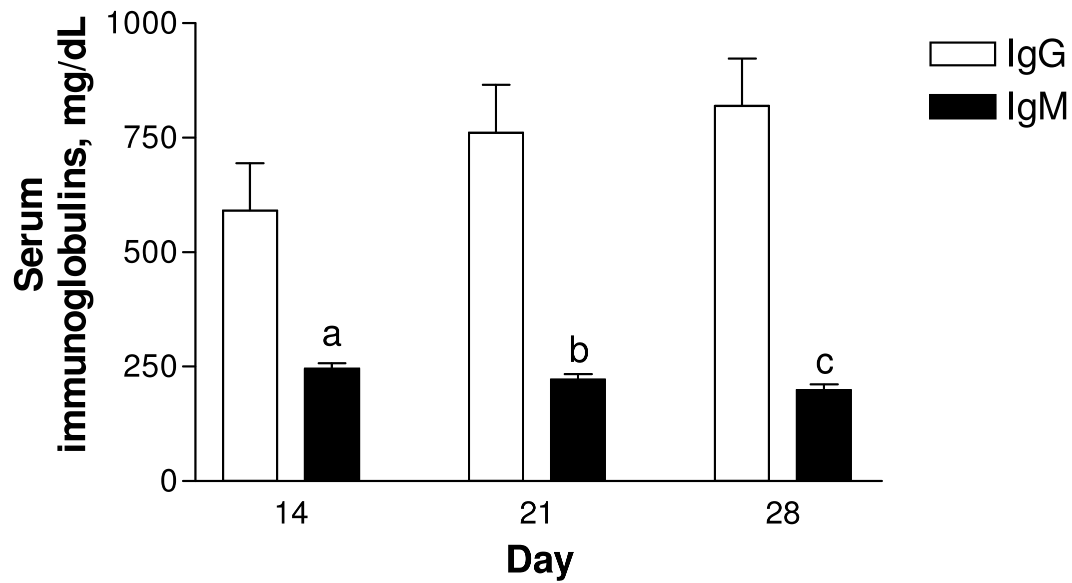


Figure 5. Serum IgG and IgM across days of the study. No significant effects of dietary treatments, Salmonella challenge, or interactions were observed. Therefore, data were pooled across all treatments. There was a tendency for a day effect for IgG ($P < .07$) and a significant effect of day for IgM ($P < .001$). For serum IgM, bars with different superscripts differ significantly ($P < .05$).

Table 1. Effects of Salmonella Challenge and Dietary ANE on Growth Performance of Weanling Pigs

	Challenge			% **ANE** in Diet				
Item	Control	Salmonella	SEM	0	.5	1.5	2.0	SEM
Day 0-7								
ADG, lb	.46	.50	.089	.43	.44	.49	.55	.071
ADFI, lb	.66	.77	.089	.60	.70	.74	.82	.110
F/G	1.52	1.59	.084	1.47	1.66	1.56	1.53	.119
Day 8-14								
ADG, lb	.94	1.04	.057	.90	1.06	1.07	.94	.071
ADFI, lb	1.22	1.36	.089	1.15	1.29	1.35	1.37	.110
F/G	1.33	1.32	.084	1.34	1.22	1.28	1.47	.119
Day 15-21								
ADG, lb	1.39[a]	.95[b]	.057	1.12	1.19	1.25	1.14	.071
ADFI, lb	1.91[a]	1.62[b]	.089	1.68	1.84	1.83	1.71	.110
F/G	1.39[a]	1.85[b]	.084	1.58	1.74	1.59	1.59	.119
Day 22-28								
ADG, lb	1.17[a]	1.40[b]	.057	1.27	1.28	1.37	1.24	.071
ADFI, lb	2.17	2.29	.089	2.14	2.20	2.24	2.34	.110
F/G	1.91[a]	1.65[b]	.084	1.74	1.81	1.62	1.94	.119

[a,b]Differ between control and Salmonella treatments ($P < .05$).

Table 2. Effects of Salmonella Challenge on Daily Feed Intake and Rectal Temperature of Weanling Pigs

Day after	Feed Intake, lb		Rectal Temperature, °F	
Challenge	Control	Salmonella	Control	Salmonella
0	--	--	103.5[a]	104.0[b]
1	1.79[a]	1.70[b]	103.6[a]	104.6[b]
2	1.77[a]	.90[b]	103.6[a]	105.2[b]
3	1.84[a]	1.18[b]	103.5[a]	104.3[b]
4	2.11[a]	1.49[b]	103.5	103.8
5	1.98	2.05	103.9	103.6
6	1.87	2.01	103.8	102.8
7	2.09	2.02	103.4	103.0

[a,b]Differ between control and Salmonella treatments ($P < .05$); Feed intake SEM ± .145; Rectal temperature SEM ± .134.