RESEARCH REPORT
PORK SAFETY

Title: Defining the health benefits of the nutritional interaction between conjugated linoleic acid and fish oil - **NPB# 02-124**

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Abstract: Synthetic peroxisome proliferator-activated receptor γ (PPAR γ) agonists are beneficial in preventing or ameliorating intestinal inflammation. We have studied the colonic health benefits of natural PPAR γ agonists such as conjugated linoleic acid (CLA) and (n-3) polyunsaturated fatty acids (PUFA) contained in fish oil. Using a model of bacterial-induced colitis, we previously demonstrated that CLA ameliorated disease associated with colitis (i.e., mucosal lesion development and weight loss) in pigs. CLA differentially modulated PPAR expression (α, γ, and δ) and PPAR γ activity in dextran sodium sulfate (DSS)-challenged mice. We hypothesized that CLA fed in combination with (n-3) PUFA would prevent colitis more effectively than CLA alone. To test this hypothesis and as a means of comparison with previous studies, we developed a pig model of DSS colitis. Sixty-four pigs were fed isocaloric diets supplemented with 1.33 g oil/100 g diet of either 1) Soybean oil (control), 2) CLA, 3) Fish oil or 4) CLA & Fish oil (50:50) for 42 days prior to the DSS challenge. On day 42, half of the pigs within dietary treatment (n=8) were administered 100 ml of 4% DSS by gastric intubation daily for 7 days. Weight loss and clinical signs were monitored on a daily basis. Colonic samples were recovered at 7 days post-challenge for transcriptional profiling of the colon and histopathological evaluation of lesions. Expression of colonic PPAR α, and PPAR γ and interferon-γ mRNA was assayed using RT-PCR. Results from the present study indicate that, while the onset of enteric disease was delayed and disease was less severe in pigs fed CLA, dietary supplementation with either (n-3) PUFA alone or (n-3) PUFA in combination with CLA resulted in clinical signs of enteric disease at 2 days, followed by recovery at 6 to 7 days post-challenge. Pigs fed the control diet were more severely affected than pigs in the other treatment groups, as measured by weight loss, colonic lesions and diarrhea. PPAR γ mRNA expression was enhanced in DSS-challenged pigs fed CLA when compared to the other treatment groups. Using the DSS model of enteric inflammation we have shown that while CLA delays the onset of disease, (n-3) PUFA accelerate the recovery. However, when CLA was fed in combination with (n-3) PUFA, the beneficial effects of CLA were diminished.

Introduction: Antimicrobial dietary supplementation in pig production increases the utilization of nutrients and the efficiency of gain by regulating changes in the microbial populations that benefit intestinal function. This explanation is supported by evidence that antimicrobials decrease intestinal wall thickness (i.e., infiltration of neutrophils and lymphocytes into the lamina propria of the intestine) (1), thus enhancing absorption of nutrients and preventing deleterious catabolic responses. Conversely, nutritional modulation targets the host rather than the gut microflora, therefore avoiding the selective pressure of indiscriminant antibiotics on a variety of bacterial genera. By favorably modulating host-microflora interactions in the intestine, dietary supplementation with polyunsaturated fatty acids (PUFA) could increase animal health and productivity.

Soybean and corn are two crucial ingredients in U.S. porcine dietary formulations. Both ingredients and their respective oils are rich in linoleic acid whose concentration in typical U.S. diets greatly exceeds the nutritional requirement for maintenance, reproduction or growth (i.e., 0.10 %/kg of diet). Linoleic acid is converted *in vivo* into arachidonic acid (2). Upon inflammatory stimulus, arachidonic acid is used as a substrate for the generation of an array of lipid mediators that is primarily pro-inflammatory (3). Thus, increased mucosal arachidonic acid levels, in the absence of the suppressive effects of growth-promoting antibiotics on the luminal microflora, will facilitate intestinal inflammation (i.e., decreased feed efficiency) in pigs. However, supplementation of antibiotic-free diets with PUFA that dampen the production of pro-inflammatory mediators and favor the endogenous production of predominantly anti-inflammatory molecules (i.e., lipid mediators and cytokines) will help overcome the adverse effects of arachidonic acid.

Conjugated linoleic acid (CLA) has been proposed as a nutritional alternative to sub-therapeutic antimicrobial usage. CLA is believed to decrease the production of arachidonic acid derivatives and to modulate the expression and activity of peroxisome proliferator-activated receptors (PPARs)(4,5). PPAR γ, α and δ belong to the superfamily of nuclear hormone receptors (6). At the molecular level, PPAR γ activation decreased the expression of pro-inflammatory cytokines (7) by antagonizing the activities of a central mediator of inflammation, namely NF-κB (8,9). While CLA enhances PPAR γ activation *in vitro* in macrophage cultures (10), linoleic acid is a poor activator of PPAR γ (11). This study investigated the ability of CLA to regulate PPAR α and γ expression *in vivo* during the onset of enteric disease.

The results of our previous research allowed us to predict immune and inflammatory outcomes associated to dietary CLA supplementation (5-7). This knowledge base can be applied to maintaining productivity and competitiveness of the U. S. pork industry after growth-promoting antibiotic usage is curtailed. **The long-term goal of this line of research is to develop nutritional alternatives to the use of sub-therapeutic antibiotics.**

It has been speculated that the (n-3) polyunsaturated fatty acids [i.e., docosahexanoic (DHA) and eicosapentanoic (EPA)] of fish oil have anti-inflammatory properties (13). The present study examined for the first time the anti-inflammatory efficacy of fish oil-derived (n-3) PUFA in the pig and examine the interactions between CLA and fish oil.

Objectives: The overall goal of this project was to develop strategies that can be applied by producers to improve swine health through natural dietary

immunomodulators and, specifically, to examine the interaction between CLA and (n-3) PUFA of fish oil to prevent intestinal inflammation.

Specific objectives

1. To examine the effects of the nutritional and immunological interactions between CLA and (n-3) fatty acids on growth performance in healthy pigs and pigs with severe intestinal inflammation.
2. To investigate the physiological changes (i.e., cytokine expression, lymphocyte profiles, and gastrointestinal function) that prevent weight losses in pigs fed CLA and fish oil-supplemented diets.
3. To make economic comparisons of the use of CLA or fish oil alone versus a 50:50 mixture to prevent weight losses caused by enteric inflammatory diseases.

Materials & Methods:

Experimental Design and Diets.

Sixty-four early-weaned pigs were randomly distributed from outcome groups based on litter, body weight, and gender into 16 blocks of 4 contiguous individual pens. Four isocaloric and isonitrogenous dietary treatments were randomly allotted to pens within a block: 1) no CLA, no supplemented (n-3) PUFA (control diet); 2) 1.33% CLA, no supplemmented (n-3) PUFA; 3) no CLA, 1.33 % (n-3) PUFA; and 4) 1.33% CLA, 1.33% (n-3) PUFA. The sources of CLA and (n-3) PUFA were Clarinol^{TM} and Marinol^{TM} (Loders Croklaan BV, Channahon, IL). During the entire experiment, pigs, feeders, and waste feed were weighed on a weekly basis to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain to feed (G:F).

Diets were formulated to be isocaloric and isonitrogenous and to meet the National Research Council (1998) (1) nutrient recommendations for swine. Pigs were given ad libitum access to feed for 7 weeks in four phases (Table 1). To maintain diets isocaloric, CLA or fish oil were replaced by soy oil in the control diet.

Induction of experimental colitis. Half of the blocks of pigs were challenged with 4 % dextran sodium sulfate (DSS) in 100 mL of water by gastric intubation once daily for 7 days (i.e., acute inflammatory challenge). The first dose of DSS water contained a volume of 200 mL and was preceded by a 12 hours fasting period. The non-challenged blocks were also fasted for 12 hours and administered the same volume of the vehicle (i.e., water) not containing DSS.

TABLE 1

Dietary Composition (as-fed basis)

Item	Control Diets[1]			
	Phase, I	Phase, II	Phase, III	Phase, IV
Ingredient, %				
Corn	32.69	49.59	63.08	72.36
Soybean meal (48 % CP)	12.00	21.20	31.00	22.00
Dried whey	22.00	17.00	—	—
Spray-dried plasma	7.50	3.00	—	—
Dried blood cells	—	2.00	0.50	—
Dry skim milk	21.00	1.50	—	—
CLA mixture[2]	—	—	—	—
(n-3) PUFA mixture	—	—	—	—
Soybean oil	2.21	2.21	2.21	2.21
DL-Methionine	0.18	0.23	0.07	0.12
L-Lysine	—	0.19	0.20	0.39
Sodium chloride	0.25	0.25	0.25	0.25
Dicalcium phosphate	1.09	1.72	1.60	1.50
Calcium carbonate	0.78	0.81	0.79	0.90
Vitamin premix[3]	0.20	0.20	0.20	0.20
Trace mineral premix[4]	0.05	0.05	0.05	0.05
Selenium premix[5]	0.05	0.05	0.05	0.05
Calculated composition, %				
Crude protein	24.36	21.17	20.30	16.43
Lysine	1.76	1.50	1.30	1.15
Methionine + cystine	0.97	0.86	0.71	0.65
Calcium	1.05	0.93	0.75	0.70
Phosphorus available	0.70	0.55	0.35	0.30
ME MJ/kg	14.43	14.15	14.25	14.27

[1] Phase I, 1-2; II, 3-4;III, 5-6; and IV, 7.

[2] In CLA-supplemented diets 2.21 % of Clarinol (containing a 50:50 mixture of cis-9, trans-11 and trans-10, cis-12 CLA) replaced 2.21 % of soybean oil to maintain the diets isocaloric within phases. In fish oil-supplemented diets, 2.21 % of Marinol (containing EPA and DHA) replaced 2.21 % soybean oil. In diets supplemented with both fish oil and CLA, 1.105 % Clarinol and 1.105 % Marinol replaced 2.21 % soybean oil.

[3] Supplied per kilogram of diet: retinyl acetate, 1,516 µg; cholecalciferol, 26 µg; dl-alpha tocopheryl acetate, 22 mg; riboflavin, 6.6 mg; pantothenic acid, 17.6 mg; niacin, 33 mg; and vitamin B-12, 22 µg.

[4] Supplied per kilogram of diet: Zn, 165 mg (ZnO); Fe, 193 mg ($FeSO_4 \cdot H_20$); Mn, 66 mg (MnO); Cu, 19.29 mg ($CuSO_4 \cdot 5\ H_20$); and I, 0.2 mg (ethylene diamine dihydroiodide).

[5] Supplied per kilogram of diet: Se, 0.1 mg (Na_2SeO_3).

Clinical evaluation, necropsy procedures and sample collection. Following the DSS challenge, clinical signs of disease were monitored on a daily basis. Pigs were necropsied by intravenous injection of sodium pentobarbital (i.e., 5 to 7 mL) on day 49 of the experiment (7 days post-challenge), the severity of colonic lesions was scored macroscopically and histologically evaluated. Sections of ileum, spiral colon and cecum were obtained, fixed in 10% buffered neutral formalin, later embedded in paraffin, sectioned (6-µm) and stained with hematoxylin and eosin (H&E) for histological examination. Samples of colon and colonic lymph nodes were embedded in RNA Later (Ambion Inc., Austin, TX) for posterior isolation of total RNA and analysis of cytokine expression (colonic samples). For immunohistochemistry, samples of colonic tissue were placed in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC) and snap frozen in liquid nitrogen. Both samples for mRNA expression analysis and for immunohistochemistry were stored at –70 °C.

Histological Evaluation of Microscopic Lesions. Tissue slides were examined in an Olympus microscope (Olympus America Inc., Dulles, VA), images were captured using the FlashBus FBG software (Integral Technologies Inc., Indianapolis, IN), and processed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Tissue sections were graded with histological scores including the extent of 1) mucosal enlargement, 2) epithelial erosion, and 3) epithelial regeneration. The sections were graded with a range from 0 to 3 for each of the previous categories. Briefly, mucosal enlargement: 0 = none; 1 =mild enlargement; 2 =moderate mucosal enlargement; 3 =severe mucosal enlargement. For regeneration: 3 = no tissue repair; 2 = surface epithelium not intact; 1 = almost complete regeneration; 0 = complete regeneration or normal tissue. For epithelial erosion: 0 =healthy epitelium; 1 = superficial erosions; 2 = multifocal ulcers; 3 = complete destruction of the epithelial layer and flattening on the epithelial cells.

Immunohistochemistry. For the evaluation of colonic lymphocyte subset distribution, frozen colonic tissue sections embedded in tissue freezing medium were cut on a cryostat at~ 18 °C at thicknesses from 5 to 10 μm. Sections were placed on poly-L-lysine coated slides, fixed in 95% methanol for 2 minutes and soaked in cryopreservative (0.5 M sucrose, 0.006 M $MgCl_2$, 50 % glycerol) for 10 minutes. Slides were stored at~ 20 °C until stained. Prior to staining tissues with monoclonal antibodies, slides were rehydrated in 0.5 M Tris Solution. Endogenous peroxidase activity was blocked by adding 0.3 % hydrogen peroxide for 10 minutes. Non-specific binding was blocked with the addition of the immunohistochemistry buffer containing 5 % Normal Goat Serum/ 3 % Bovine Serum Albumin/Tris buffer (NGS/BSA/Tris) solution at room temperature for 2 hours. Slides were incubated with the primary antibody solution overnight at 4 °C. Primary antibodies were diluted in NGS/BSA/Tris. Mouse anti-pig CD4 primary antibody will be 10 × supernatant from the mouse cell line HB147 used at 1:25 dilution. Mouse anti-pig CD8α antibody was supernatant from the mouse cell line HB143 used at 1:100 dilution. Both antibodies were generated in our laboratory from the HB143 and HB147 cells lines obtained from ATCC. The mouse anti-pig TCRδ (PGBL22A) antibody was purchased from VMRD (Pullman, WA) and used at 1:100 dilution. The mouse anti-pig CD3 was concentrated supernatant from the mouse cell line 8E6 and used at 1:10,000 dilution.

Prior to the incubation with the secondary antibodies, slides were rinsed with Tris solution to wash unbound primary antibody. Peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) was added to slides stained with: mouse anti-pig CD4, mouse anti-pig CD3, and mouse anti-pig TCRδ. The goat anti-mouse IgG (H + L) was diluted 1:300 with NGS/BSA/Tris and incubated for 2 hours (RT). For the mouse anti pig CD8α primary antibody, the secondary used were biotin-conjugated goat $F(ab')_2$ anti-mouse IgG2a (Southern Biotechnologies Associates Inc, Birmingham, AL) diluted 1:250 (in NGS/BSA/Tris), incubated for 2 hours at RT. Following the second incubation, slides were treated with peroxidase-conjugated strepavidin, diluted in Tris solution (1:500) and incubated for 1 hour (RT). The chromagen utilized was diaminobenzediene (Biomedia Corporation, Foster City, CA). Slides were counterstained with Instant Hematoxylin (Shandon, Pittsburg, PA), coversliped with Immu-mount (Shandon) and numbers of $CD4^+$, $CD8^+$, $CD3^+$ and $TCR\delta^+$ cells enumerated. Stained colonic sections were observed at 400 × magnification. Five randomly chosen sections (i.e., area 0.375 mm^2) were enumerated

for each pig and antibody treatment. Data were presented as number of cells per square millimeter.

Regulation of gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR).

Colonic tissue recovered during the necropsy procedure was kept in RNAlater™ (Ambion, Austin, TX) at −70 °C. Total RNA was isolated using the total RNA isolation MiniKit (Qiagen, Valencia, CA), treated with DNA-freeTM (Ambion), and kept in 0.02% diethyl pyrocarbonate (DEPC)-treated water at –20°C according to the manufacturer's instructions. RNAs in samples were quantified, and the purity determined using a spectrophotometer at an optical density $(OD)_{260}$ and OD_{260}/OD_{280} ratios, respectively. All samples had OD_{260}/OD_{280} ratios above 1.80 corresponding to 90–100% pure nucleic acid.

In order to assess the nutritional regulation of endogenous anti-inflammatory pathways (i.e., PPAR γ) by CLA colonic sections were collected during the necropsy. These samples were appropriately prepared and assayed for the relative expression of PPAR α and PPAR γ normalized to β-actin by using RT-PCR. Briefly, following isolation of total RNA, one microgram of each RNA isolate from each pig was added into a 5-μL DNA digestion reaction containing 4 μL of M-MLV RT reaction buffer (Promega, Madison, WI), 0.4 μL of RNase-free water, 0.5 μL of SUPERase In (Ambion, Austin, TX), and 0.1 μL of Dnase I (Sigma, St Louis, MO). Cycle parameters for DNA-digestion were 1 cycle of 37°C, 15 min; 1 cycle of 94°C, 10 min; 1 cycle of 4°C, 5 min. For melting of the secondary structure, 1 μL of Promega random hexamers were added into the digested RNA. Cycle parameters for the melting reaction were 1 cycle of 94°C, 5 min. After the melting reaction, the reaction mixture was placed onto ice for 1 min. RNA was then reverse transcribed in a 10-μL reaction containing 6 μL of the previously described reactions plus 1 μL of M-MLV RT reaction buffer (Promega, Madison, WI), 1.25 μL Sigma dNTP mix, 0.75 μL of RNase-free water, and 1 μL of Promega M-MLV RT (200 U reverse transcriptase/μL). Cycle parameters for the reverse transcription procedure were 1 cycle of 37°C, 60 min; 1 cycle of 94°C, 5 min; and 1 cycle of 4°C, 5 min. The entire 10-μL reaction was then subjected to PCR amplification in a PCR reaction with a total volume of 50 μL containing 3 μL of Promega 25 mM $MgCl_2$, 4 μL of Gibco PCR buffer without $MgCl_2$, 35.5 μL of PCR water, 1 μL of forward primer, 1 μL of reverse primer, and 0.5 μL of Taq polymerase (Life Technologies, Inc., Rockville, MD). Cycle parameters for PCR amplification were 1 cycle of 94°C, 2.5 min; 32 cycles of (94°C, 1 min; 55°C, 1 min; 72°C, 1 min); 1 cycle of 72°C, 10 min; and 1 cycle of 4°C, 5 min. Each cDNA pool was amplified using primers specific for the target gene. PCR-amplified products were electrophoretically separated on a 1.5% agarose gel. A 100-Kbp ladder (100 Kbplus, Life Technologies) was used as size standard.

Statistical Analysis. Prior to the DSS challenge data was analyzed as a randomized complete block design. Post-DSS challenge, data was analyzed as a split-plot design, with each pig within a block being the experimental unit for dietary treatment (sub-plot) and 8 blocks of 4 littermate pigs within DSS challenge status being the experimental unit for DSS challenge (whole plot). More specifically, data was analyzed as a 4×2 factorial arrangement of treatments (4 diets and 2 DSS states) within a split-plot design with sixteen blocks of four littermate pigs as the experimental unit for DSS treatment (i.e., non-challenged or challenged with DSS) and pig within block as the experimental

unit for dietary treatment (soy oil, conjugated linoleic acid, fish oil or CLA & fish oil). The whole plot error (i.e., error A) being block within DSS status (i.e., 14 degrees of freedom) and the sub-plot error (i.e., error B) being the residual degrees of freedom after accounting for the dietary treatment variance and the variance for the interaction between diet and DSS treatment (i.e., 42 degrees of freedom). Analysis of variance after DSS challenge was performed using the general linear model (GLM) procedure of SAS using the TEST statement (14). A $p < 0.05$ was considered statistically significant. The statistical model utilized was $Y_{ijk} = \mu + DSS_i + \text{error } A_{ik} + Diet_j + (DSS \times Diet)_{ij} + \text{error } B_{ijk}$, μ being the general mean, DSS_i being the main effect of the i_{th} level of the DSS effect, $Diet_j$ being the main effect of the j_{th} level of the dietary effect, $(DSS \times Diet)_{ij}$ being the interaction effect between DSS and diet, and errors A and B representing the random errors for the whole plot and the sub-plot, respectively.

Results:

DSS titration study

Two preliminary experiments were designed to determine the optimal dose of the DSS (i.e., 0, 1.25, 2.5 or 5%) treatment. The initial experiment indicated that 2.5% DSS added in the drinking water provided an optimal response (i.e., clinical signs, weight loss, and lesion development). The second experiment examined the effects of (0, 2, 4, or 6% DSS in 100 mL of water). The results indicated that optimal DSS responses could be also achieved by gastric intubation of pigs using 4% DSS in 100 mL of water on a daily basis for 7 days. We administered 4% DSS by gastric intubation in the main feeding trial.

Growth performance

Prior to the DSS challenge, no differences in ADG, ADFI or G:F were found between dietary treatments. Following the DSS challenge, an interaction between DSS and dietary treatment was found for ADG and G:F ($P < 0.03$ and 0.003, respectively). Overall, the ADG was lower in DSS challenged pigs than in non-challenged pigs. However, the growth suppression was attenuated in CLA-fed pigs. When both CLA and fish oil were added, the fish oil overshadowed the positive effects of CLA on growth suppression. G:F in challenged pigs fed CLA was 85% of the G:F of non-challenged pigs. Conversely, the G:F of pigs fed the control diet, fish oil, or the combination of CLA and FO diet were at 23, 33 and 55% of the G:F in non-challenged pigs, respectively (Table 2).

Clinical Signs of Disease Associated with Enteric Inflammation

The major clinical signs of disease associated to the DSS challenge were diarrhea, abdominal pain, and lethargia. The signs of disease became apparent at day 3 after the DSS challenge, and the enteric disease outbreak, as measured by fecal scores, peaked between days 5 and 7 depending on the dietary treatments. No signs of disease were observed in pigs not challenged with DSS. The onset of enteric disease was accelerated in pigs fed fish oil either alone or in combination with CLA (Figure 1). Fish oil-fed pigs recovered clinically on day 7 of the DSS challenge. The onset of disease in pigs fed the control diet (i.e., soybean oil-supplemented) was delayed but the clinical signs indicated a greater disease severity than the other treatment groups. Dietary CLA-supplementation alone was the most effective treatment in delaying the onset of enteric disease and attenuating the clinical signs during the 7-day DSS challenge. Macroscopically, the ileum and the colon were the portion of the

gastrointestinal tract most affected by the DSS challenge. Colon and ileum presented signs of mild to severe irritation. However, no significant differences in macroscopic lesion scores were found between treatment groups.

Histology

The response to the DSS treatment consisted on mucosal enlargement and epithelial erosion with or without regeneration. The severity of the mucosal enlargement and epithelial erosion in pigs fed the diet supplemented with CLA only, was lower than in the other three treatment groups ($P < 0.002$ and 0.001, respectively). Signs of epithelial regeneration such as an enhanced presence of mitotic figures in epithelial cells was greater in pigs fed fish oil supplemented diets, regardless of the presence of CLA in the diet (Table 3).

Lymphocyte Profiles

$\gamma\delta$ T cells contribute to tissue regeneration through the production of epithelial growth factors. No major differences in $\gamma\delta$ T cell numbers in the colon or peripheral blood were attributable to dietary treatment in pigs not challenged with DSS. Pigs fed fish oil-supplemented diets had a significant increase of colonic $\gamma\delta$ T cells following the DSS challenge (51.99/mm^2 in non-DSS versus 70.16/mm^2 in DSS-challenged pigs fed fish oil, and 56.17/mm^2 in non-DSS versus 68.17/mm^2 in DSS-challenged pigs fed fish oil and CLA, $P < 0.05$). These numbers of $\gamma\delta$ T cells in fish oil-fed pigs were positively correlated with increased epithelial regeneration. The effects of CLA alone on modulating $\gamma\delta$ T cells kinetics were very limited (Table 4).

Expression of Cytokines and Peroxisome Proliferator-Activated Receptors α and γ

We examined the mRNA expression of PPAR α and γ by RT-PCR. PPAR δ has not been cloned and sequenced in the pig. We found that following the DSS challenge the expression of PPAR γ was suppressed in pigs with more severe DSS colitis (i.e., fed control or fish oil supplemented diets). However, the PPAR γ expression levels in DSS-challenged pigs fed CLA were similar to those of non-challenged pigs. The greater expression of PPAR γ mRNA in CLA-fed pigs was accompanied with lower PPAR α expression mRNA levels (Figure 2). The greater expression of PPAR γ and lower levels of PPAR α mRNA correlated with lower levels of INF-γ in the colons of CLA-fed pigs following the DSS challenge (Figure 2).

Discussion: The effects of CLA on feed efficiency were not consistent from one pig feeding trial to another. We proposed that subclinical infections and adverse inflammatory responses could account for the lack of consistency between experiments. Our previous bacterial infection study (4) demonstrated that CLA improved G:F in pigs challenged with an enteric bacterial pathogen. The present study used a broader inflammatory stimulus to examine the interactions between CLA and (n-3) PUFA of fish oil.

In contrast to what it was anticipated, we found no nutritional or immunological synergic effects between CLA and fish oil. Fish oil diminished the beneficial effects of CLA in G:F and lesion development during the onset of enteric disease. While CLA enhanced PPAR γ mRNA expression in the colonic mucosa of DSS challenged pigs, the control diet or fish oil-supplemented diets enhanced PPAR α. Pigs that were fed both CLA and (n-3) PUFA also enhanced PPAR γ expression without PPAR α repression but not PPAR γ activity. In this regard, (n-3) PUFA are potent PPAR γ ligands (22) but

suppress PPAR γ transcriptional activity (23). These molecular changes provide a very likely explanation for the results of this study.

In a previous final report submitted by the Laboratory of Nutritional Immunology & Molecular Nutrition to the National Pork Board in September of 2002 (#01134) we summarized the results of a study conducted by Pettigrew in 1999. This study was the first economic analysis resulting from a series of earlier CLA feeding trials in pigs (15). This initial report analyzed the economic benefits for enhanced feed efficiency (e.g., savings of 99 grams of feed per kg of gain) and carcass leanness in finishing pigs. The report concluded that based on these two performance criteria and depending on the leanness premium and degree of sophistication of the nutritional program, the total economical advantage of feeding CLA would range between $3.16 and $4.62 per pig. However, a number of pig feeding trials demonstrate that CLA does not influence G:F in healthy pigs (4,12,16-20). Hence, these benefits may not be of relevance. Dr. Pettigrew's report indicated that the economic value for increased immune function, if any, could not be estimated at the time the study was completed due to a lack of published results on the health benefits of feeding CLA.

Based on the results of a bacterial infection study, we concluded that the economical benefits associated to G:F and immunomodulation improvements during a bacterial infection could reach $6.72 and 1.52 per pig, respectively. In the bacterial-induced colitis model, the G:F of infected pigs fed CLA represented 86% of the G:F in non-infected pigs fed CLA. Herein, in similar action, the G:F of DSS-challenged pigs fed CLA represents 85% of the non-DSS challenged pigs. Thus, the conclusions that we can be draw from this study resemble those of the previous report. These economical benefits could be greater if food safety considerations were included in the economical analysis. However, the efficacy of CLA has never been compared directly to sub-therapeutic antibiotics. Thus, additional research is needed to provide a more complete picture of CLA's benefits.

An additional objective of the present study was to examine the economical benefits of the interaction between CLA and fish oil. In contrast to what it was anticipated, the clinical, pathological and growth performance results indicate that fish oil does not prevent inflammation associated to the DSS challenge. Consequently a positive interaction between CLA and fish oil on inflammation was not found. Furthermore, when CLA was fed in combination with fish oil, the beneficial effects of CLA were diminished. No additional economical benefit can be attributed to supplementing diets with both CLA and fish oil.

When Pettigrew's report and our previous report were completed, livestock quality grade CLA was not readily available. CLA is now available commercially for livestock production. The prices range from $5 to $13 per kilo of CLA. Specifically, BASF AG has a CLA product in the market that is sold for $12 to $13/kg. There are indications that Loders Croklaan BV, the major CLA manufacturer, will sell livestock quality grade CLA for $5/kg. When compared to the anticipated benefits, the latter prices indicate that feeding CLA at critical phases of the pig's development may be economically feasible. Targeting the phases of dietary CLA supplementation would maximize the impact on immune function while minimizing the total amount of CLA needed. In this regard, we have reported that the effects of CLA on immune function are long-lived and persist beyond the period of dietary supplementation (21). Based on our experience, adding CLA in diets of phase I and II could maximize the immunomodulatory effects of this compound. The optimal CLA concentration for optimal immunological impact is 1.3 % in the diet (12). By adding 1.3% CLA during phases I and II we guarantee that the CLA intake per pig throughout production will remain below 1

kilogram (i.e., 870 grams). Assuming a CLA cost of $5/kg, the cost of adding CLA in the diet could be offset by the G:F improvements during a bacterial infection.

Summary of the knowledge of benefit to producers

CLA improves G:F in pigs with gastrointestinal disease. Supplementing pig dietary formulations with CLA at 1.33% could be economically feasible in those operations prone to intestinal disease outbreaks and/or other infectious diseases with a strong inflammatory component. Dietary supplementation of pig diets with both CLA and fish oil is not recommended because the fish oil diminishes the beneficial effects of CLA. To maximize profits associated with dietary CLA supplementation, no more than 1 kg of CLA per pig is recommended throughout the entire production period. CLA should be administered at very early stages of development (Phases I and II).

Lay Interpretation: Sub-therapeutic antibiotics attenuate bacterial growth and intestinal inflammation. These positive effects of anti-microbial agents result in increased feed efficiency and reduced time to market. The development of anti-microbial resistant bacteria pathogenic to humans has been linked to adding sub-therapeutic antimicrobials in pig dietary formulations. For instance, according to government surveys, most *Salmonella* isolates recovered from swine are antibiotic resistant. The results of this and previous studies from our laboratory demonstrate that, in similar action to antibiotics, conjugated linoleic acid (CLA) ameliorates intestinal inflammation in pigs. In contrast to sub-therapeutic antibiotics, CLA is not linked to the development of antibiotic-resistant bacteria. Hence, CLA may be a viable nutritional alternative to sub-therapeutic anti-microbial usage. This project has demonstrated that the anti-inflammatory effects of fish oil are very limited when compared to CLA. Furthermore, when both CLA and fish oil are added, the fish oil interferes with the anti-inflammatory effects of CLA. The economical benefits of dietary CLA-supplementation associated to G:F improvements during a bacterial infection average $6.72 per pig. These benefits can offset the initial cost of adding CLA in the diet (i.e., $5/pig). The next step of our line of research involves direct comparisons of the effects of CLA versus sub-therapeutic antibiotics in commercial hog operations. If the National Pork Board funded this line of research, the economical benefits related to food safety could be also calculated. Contact information: Josep Bassaganya-Riera DVM, PhD, Nutritional Immunology & Molecular Nutrition Laboratory, 253 Wallace Hall, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, phone (540) 231-7421, email: jbassaga@vt.edu.

References:

1. National Research Council (1998) Nutrient requirements of swine. National Academy Press, Washington, D. C.
2. Calder, P. C. (1998) Dietary fatty acids and the immune system. Nutr Rev 56: S70-83.
3. Sharon, P., Ligumsky, M., Rachmilewitz, D. & Zor, U. (1978) Role of prostaglandins in ulcerative colitis. Enhanced production during active disease and inhibition by sulfasalazine. Gastroenterology 75: 638-640.
4. Hontecillas, R., Wannemeulher, M. J., Zimmerman, D. R., Hutto, D. L., Wilson, J. H., Ahn, D. U. & Bassaganya-Riera, J. (2002) Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid. J Nutr 132: 2019-2027.
5. Hontecillas, R., Jobgen, S. C., Wannemeulher, M. J. & Bassaganya-Riera, J. (2003) Differential regulation of colonic PPAR expression by dietary fatty acids. Experimental Biology 2003, San Diego, CA.

6. Wolf, G. (1998) Fatty acids bind directly to and activate peroxisome proliferator- activated receptors alpha and gamma. Nutr Rev 56: 61-63.
7. Jiang, C., Ting, A. T. & Seed, B. (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391: 82-86.
8. Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J. & Glass, C. K. (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391: 79-82.
9. Desreumaux, P., Dubuquoy, L., Nutten, S., Peuchmaur, M., Englaro, W., Schoonjans, K., Derijard, B., Desvergne, B., Wahli, W., Chambon, P., Leibowitz, M. D., Colombel, J. F. & Auwerx, J. (2001) Attenuation of Colon Inflammation through Activators of the Retinoid X Receptor (RXR)/Peroxisome Proliferator-activated Receptor gamma (PPARgamma) Heterodimer. A basis for new therapeutic strategies. J Exp Med 193: 827-838.
10. Yu, Y., Correll, P. H. & Vanden Heuvel, J. P. (2002) Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta 1581: 89-99.
11. Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M. & Lazar, M. A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J Biol Chem 270: 23975-23983.
12. Bassaganya-Riera, J., Hontecillas, R., Bregendahl, K., Wannemuehler, M. J. & Zimmerman, D. R. (2001) Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition, and immune competence. J Anim Sci 79: 714-721.
13. Serhan, C. N., Clish, C. B., Brannon, J., Colgan, S. P., Chiang, N. & Gronert, K. (2000) Novel Functional Sets of Lipid-derived Mediators with Antiinflammatory Actions Generated from Omega-3 Fatty Acids via Cyclooxygenase 2-Nonsteroidal Antiinflammatory Drugs and Transcellular Processing. J Exp Med 192: 1197-1204.
14. SAS (1988) SAS/STAT User's guide (Release 6.0.3). SAS Inst. Inc., Cary, NC.
15. Pettigrew, J. E. (1999) An economic analysis of feeding conjugated linoleic acid to finishing pigs. 30th Annual Meeting of the American Association of Swine Practitioners, St. Louis, Missouri.
16. Bassaganya-Riera, J., Hontecillas, R., Zimmerman, D. R. & Wannemuehler, M. J. (2001) Dietary conjugated linoleic acid modulates phenotype and effector functions of porcine cd8(+) lymphocytes. J Nutr 131: 2370-2377.
17. Bassaganya-Riera, J., Hontecillas, R., Hutto, D. L., Zimmerman, D. R. & Wannemuehler, M. J. (2001) Dietary conjugated linoleic acid attenuates intestinal inflammation. Proceedings of the Conference of Research Workers in Animal Diseases.
18. Bassaganya-Riera, J., Hontecillas, R., Zimmerman, D. R. & Wannemuehler, M. J. (2002) Long-term influence of lipid nutrition on the induction of CD8+ responses to viral or bacterial antigens. Vaccine 20: 1435-1444.
19. Bassaganya-Riera, J., Hontecillas, R. & Beitz, D. C. (2002) Colonic anti-inflammatory mechanisms of conjugated linoleic acid. Clinical Nutrition 21: 451-459.
20. Bassaganya-Riera, J., Hontecillas, R., Wannemuehler, M.J. (2001) Nutritional Impact of Conjugated Linoleic Acid: A Model Functional Food Ingredient. In Vitro Cellular and Developmental Biology 38: 241-246.
21. Bassaganya-Riera, J., Hontecillas, R., Zimmerman, D. R. & Wannemuehler, M. J. (2002) Long-term influence of lipid nutrition on the induction of CD8(+) responses to viral or bacterial antigens. Vaccine 20: 1435-1444.
22. Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Willson, T. M., Kliewer, S. A. & Milburn, M. V. (1999) Molecular recognition of fatty acids by peroxisome proliferator- activated receptors. Mol Cell 3: 397-403.
23. Lee, J. Y. & Hwang, D. H. (2002) Docosahexaenoic acid suppresses the activity of peroxisome proliferator-activated receptors in a colon tumor cell line. Biochem Biophys Res Commun 298: 667-674.

TABLE 2

Effect of dietary treatments of pigs fed diets supplemented with soybean oil, conjugated linoleic acid (CLA), fish oil or CLA & fish oil on growth performance following a 7-day challenge with dextran sodium sulfate (DSS)[1, 2, 3]

Item	Diets (D)	Non-challenged pigs				DSS challenged pigs[4]				SEM	*P* value[5]
		Control	CLA	Fish Oil	CLA & Fish Oil	Control	CLA	Fish Oil	CLA & Fish Oil		Interaction D × DSS
ADG, g											
7th week		711[a]	727[a]	700[a]	697[a]	110[e]	453[b]	195[d]	300[c]	92	0.03
ADFI, g											
7th week		1,344	1,363	1,295	1,284	880	987	1,071	1,002	65	0.28
G:F											
7th week		529[a]	533[a]	540[a]	542[a]	125[d]	458[a]	182[c]	299[b]	79	0.003

[1] Pigs, feeders, and waste feed were weighed on a weekly basis, and average daily gain (ADG), average daily feed intake (ADFI), and gain to feed (G:F) were calculated.

[2] Least squares means values (n = 8) in a row for each growth performance criteria with different superscripts are significantly different ($P < 0.05$).

[4] On day 42, eight blocks of 4 pigs each were challenged with 4 % DSS. All experimentally challenged pigs were monitored for clinical signs of enteric disease.

[5] Following challenge, data were analyzed as a 2 × 4 factorial arrangement (i.e., 2 DSS status and 4 dietary treatments) within a split-plot design. DSS status represented the whole plot and dietary treatments the sub-plot. The experimental unit for the whole plot was a block of 4 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-value represents the interaction between the DSS status and the dietary treatments.

TABLE 3

Effect of dietary treatments of pigs fed diets supplemented with soybean oil, conjugated linoleic acid (CLA), fish oil or CLA & fish oil on colonic lesions and tissue regeneration following a 7-day challenge with dextran sodium sulfate (DSS)[1, 2, 3]

Item	Diets (D)	Non-challenged pigs				DSS challenged pigs[4]				SEM	*P* value[5]
		Control	CLA	Fish Oil	CLA & Fish Oil	Control	CLA	Fish Oil	CLA & Fish Oil		D×DSS
Thickness		0.00 [b]	0.00 [b]	0.00 [b]	0.00 [b]	2.12 [a]	0.25 [b]	2.00 [a]	1.87 [a]	0.21	0.0002
Erosion		0.00 [b]	0.00 [b]	0.00 [b]	0.00 [b]	1.87 [a]	0.00 [b]	0.12 [b]	0.25 [b]	0.17	0.0001
Regeneration		0.00 [b]	0.00 [b]	0.00 [b]	0.00 [b]	0.00 [b]	0.25 [b]	2.75 [a]	2.75 [a]	0.10	0.0001

[1] On d 49 of the experiment pigs were killed and colonic samples were formalin-fixed, embedded in paraffin and stained with hematoxylin and eosin.

[2] Least squares means values (n = 8) in a row for each growth performance criteria with different superscripts are significantly different ($P < 0.05$).

[4] On day 42, eight blocks of 4 pigs each were challenged with 4 % DSS. All experimentally challenged pigs were monitored for clinical signs of enteric disease.

[5] Following challenge, data were analyzed as a 2 × 4 factorial arrangement (i.e., 2 DSS status and 4 dietary treatments) within a split-plot design. DSS status represented the whole plot and dietary treatment the sub-plot. The experimental unit for the whole plot was a block of 4 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-values represent the interaction between the DSS status and the dietary treatments.

TABLE 4

Effect of dietary treatments of pigs fed diets supplemented with soybean oil, conjugated linoleic acid (CLA), fish oil or CLA & fish oil on total numbers of γδ Tcells in blood and the colonic mucosa following a 7-day challenge with dextran sodium sulfate (DSS)[1, 2, 3]

Item	Diets (D)	Non-challenged pigs				DSS challenged pigs[4]				SEM	*P* value[5]
		Control	CLA	Fish Oil	CLA & Fish Oil	Control	CLA	Fish Oil	CLA & Fish Oil		D × DSS
Blood											
D 49		5.17[a]	5.30[a]	5.00[a]	6.12[a]	5.45[a]	4.79[a]	3.22[b]	2.91[b]	0.62	0.03
Colon											
D 49		54.67[a]	53.28[a]	51.99[a]	56.17[a]	50.12[a]	55.23[a]	70.16[b]	68.17[b]	26	0.005

[1] Pigs were bled by vena cava puncture on day 49 of the experiment. Whole blood diluted in PBS and stained with monoclonal antibodies against TCRδ. Erythrocytes were lysed after the staining. Peripheral blood data from the flow cytometric evaluation is presented as numbers of $\gamma\delta^+$ cells (10^6/mL blood). Colonic data is presented as number of $\gamma\delta^+$ cells/mm^2 of colon.

[2] Least squares means values (n = 8) in a row for each growth performance criteria with different superscripts are significantly different ($P < 0.05$).

[4] On day 42, eight blocks of 4 pigs each were challenged with 4 % DSS. All experimentally challenged pigs were monitored for clinical signs of enteric disease.

[5] Following challenge, data were analyzed as a 2 × 4 factorial arrangement (i.e., 2 DSS status and 4 dietary treatments) within a split-plot design. DSS status represented the whole plot and dietary treatments the sub-plot. The experimental unit for the whole plot was a block of 4 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-value represents the interaction between the DSS status and the dietary treatments.

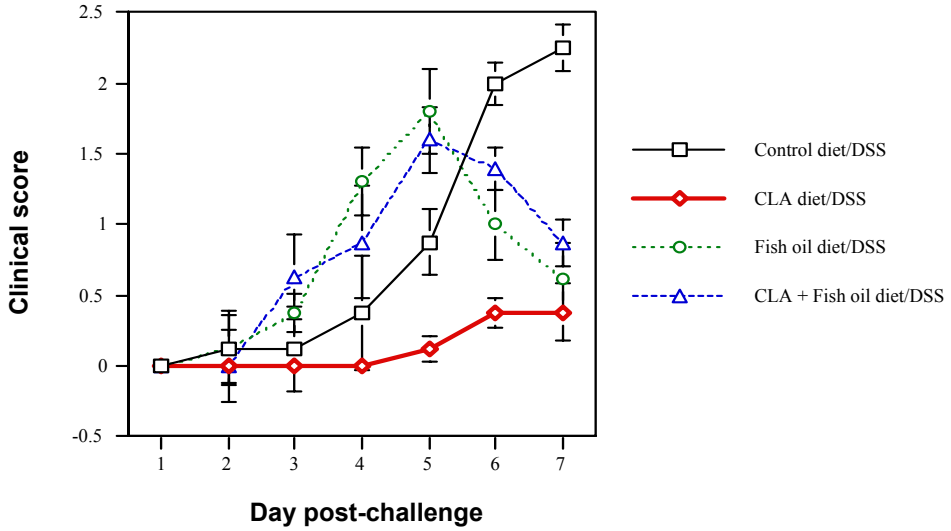

Figure 1. Clinical score of pigs challenged with 4% dextran sodium sulfate by gastric intubation. Clinical signs of enteric disease appeared as early as 3 days after challenge. Disease was more severe in pigs fed diets supplemented with soybean oil (control), fish oil, or CLA & fish oil. Dietary CLA-supplementation delayed the onset of disease and attenuated disease severity. Recovery from disease was observed in pigs fed fish oil supplemented diets.

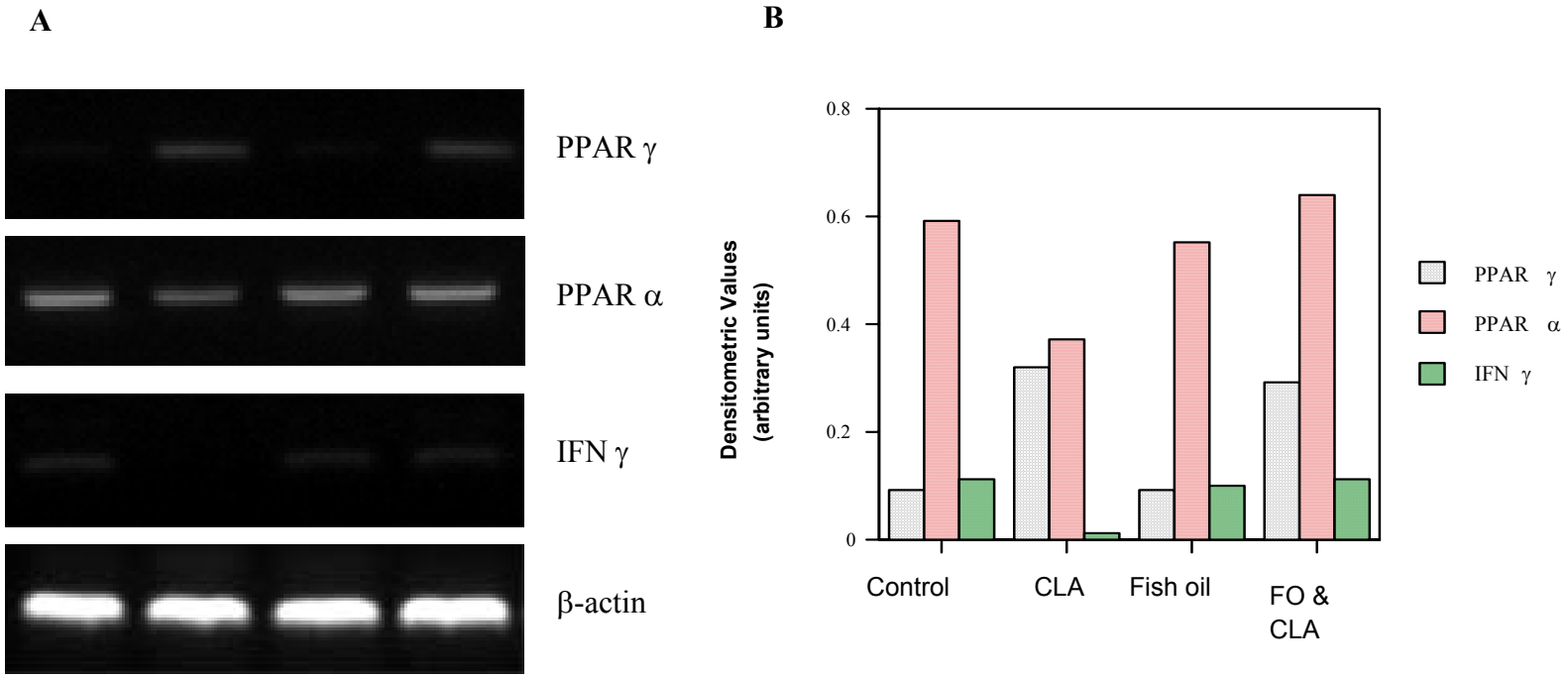

Figure 2. Peroxisome proliferators-activated receptor (PPAR) α and γ and interferon (IFN)-γ mRNA expression in the colonic samples recovered from pigs challenged with 4% dextran sodium sulfate during 7 days. The internal standard is represented by β-actin. (A) Lane 1: control diet, lane 2: CLA diet, lane 3: fish oil (FO) diet, and lane 4: CLA & fish oil diet. The RT-PCR analysis was conducted considering the original blocks of four pigs. (B) PPAR αand γ and IFN-γ expression levels were determined semiquantitatively by calculating the ratio of densitometric value of each PCR product in relation to the density of the internal standard represented by β-actin.