RESEARCH REPORT
PORK SAFETY

Title: Conjugated Linoleic Acid and Vitamin A: A Nutritional Therapy for Post-weaning Multisystemic Wasting Syndrome **NPB# 01142**

Investigator: J. Bassaganya-Riera DVM, PhD

Institution: Nutritional Immunology & Molecular Nutrition Laboratory
Department of Food Science and Human Nutrition
Iowa State University

Co-Investigators: R. Hontecillas, M. J. Wannemuehler, P. G. Halbur, D. U. Ahn, K-J. Yoon, and R. Pogranichniy

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Abstract

Dietary CLA-supplementation enhanced antigen-specific proliferation of porcine $CD8^+$ T cells to viral antigens following vaccination of pigs with a modified-live pseudorabies virus vaccine. Challenge studies using infective viruses, however, are necessary to investigate if nutritionally derived immune enhancement results in increased protection from viral disease. The present study used a viral infection model [i.e., type 2 porcine circovirus (PCV2)] to examine the effects of dietary CLA-supplementation on virally induced lymphoid depletion and immune suppression. We hypothesized that PCV2-associated disease can be ameliorated by feeding CLA prior to the viral challenge. To test this hypothesis, following 42 days of dietary supplementation with either soybean oil or CLA, viral disease was induced by challenging pigs with PCV2. A factorial (2×3) arrangement within a split-plot design with 16 blocks of three littermate pigs as the experimental unit for infective status (i.e., infected with PCV2 or non-infected) and pig within block as the experimental unit for dietary treatment (i.e., control, CLA, or CLA & 10 fold Vitamin A). Lymphoid depletion of the lymph nodes and interstitial pneumonia in the lungs was macroscopically and histopathologically evaluated. The phenotype of the depleted lymphocyte subsets was characterized by flow cytometry. Proliferation of lymphocytes in response to *ex vivo* stimulation with a recombinant capsid protein open reading frame 2 (ORF2) of PCV2 was assessed by PKH67 and blastogenesis assays. Serum samples were assayed for the presence of ORF2-specific antibodies using an indirect enzyme-linked immunosorbent assay and IFA. Dietary CLA-supplementation enhanced the *ex vivo* proliferative responses of peripheral blood and lymph node mononuclear cells to ORF2 antigens. Lymphocytes with enhanced antigen-specific proliferative abilities in CLA-fed pigs were primarily $CD8^+$ T cells. In contrast to the enhancement of T cell responses, B cell effector functions were either maintained or decreased as shown by the lower concentrations of PCV2-specific antibodies in infected

pigs fed CLA. These immunological changes resulted in a lower lymphocyte depletion (i.e., B cells) in peripheral blood, and lower growth suppression in CLA-fed pigs following infection.

Introduction

Cytotoxic T lymphocytes and natural killer (NK) cells are critical elements for the development of antiviral immunity. Depletion of $CD8^+$ cytotoxic T lymphocytes interferes with development of effective immune responses to simian/human immunodeficiency virus (1). Our laboratory has demonstrated for the first time that dietary CLA expands peripheral blood cytotoxic T lymphocytes (CTL), TCR□□CD8□□ lymphocytes, and NK cells in pigs (2-4). Vitamin A has also been shown to impact cellular immunity by enhancing CTL (5) and NK cell function (6). Moreover, a link between the effects of vitamin A on cellular immunity and health was demonstrated in chickens. It was shown that dietary-induced vitamin A deficiency impairs CTL activity in chickens infected with Newcastle disease virus (5). In addition, a common denominator in most National Research Council (NRC) publications on nutrient requirements of animals is the immunoregulatory role of vitamin A (Table 1).

TABLE 1

Immunomodulatory nutrients based on NRC publications.

The nutrient requirements		
Years	Species	Nutrients with immunomodulatory effects
1994	Poultry	Vitamin A, pyridoxine, n-3 / n-6 PUFA
1988 and 1998	Swine	Vitamin A, riboflavin, folacin, panthotenate,
1996	Beef cattle	Vitamin A, selenium, zinc, cobalt
1989	Horses	Vitamin A, pyridoxine, vitamin E
1995	Lab. Animals	Vitamin A, folacin, niacin, panthotenate, zinc
1985	Dogs	Vitamin A, folacin
1993	Fish	Vitamin A, vitamin E, vitamin C*
1989	Dairy cattle	Vitamin A
1981	Goats	Vitamin A

Only those nutrients that, based on the respective NRC references, have been reported to elicit direct effects on immune response or on cells of the immune system were included.

* Indicates that NRC reported that dietary immunomodulation was demonstrated to be non-existent for this nutrient.

Vitamin A activity has been linked to a peroxisome proliferator activated receptor-γ (PPAR-γ)/ retinoid X receptor-□ (RXR-□)-dependent signaling pathway (7). RXR-specific ligands are capable of promoting typical PPAR-γ activities (8), such as macrophage differentiation, in cultured cells given either vitamin A alone or in combination with PPAR-γ ligands (e.g., polyunsaturated fatty acids; CLA). In addition, modulation of cellular homeostasis by dietary fatty acids may also occur through transcriptional regulation of gene expression (9-11). Polyunsaturated fatty acids are known to be natural PPAR-γ ligands (7). Thus, the interaction between a PPAR-γ ligand and a RXR ligand, during the onset of viral diseases, may provide a nutritional value for health that goes beyond the basic nutritional requirements of the pig for maintenance, growth or reproduction.

For the last three years, our research group at Iowa State University has investigated the effects of CLA on immune function and health of pigs. We demonstrated that after feeding CLA for at least 42 days, numbers of peripheral blood $CD8^+$ T cells increase. Also, upon dietary CLA withdrawal, pigs that had been fed CLA had greater $CD8^+$ cells for 67 days after dietary CLA withdrawal (4). Similarly, vitamin A influences functions mediated by $CD8^+$ cells (5). Because both CLA and vitamin A influence numbers and function of cells involved in antiviral responses, the interaction between both compounds could prepare the immune system to fight against viral infections.

The porcine circovirus type 2 (PCV2), along with porcine parvovirus (PPV) has been shown to replicate in postweaning multisystemic wasting syndrome (PMWS)-affected pigs. Both PCV2 and PPV contribute to the pathogenesis of this emerging disease that becomes clinically apparent between 6 and 8 weeks of age. More recently, it has been demonstrated that some of the hallmark lesions found in PMWS can be reproduced by challenge of pigs with PCV2 alone (12). Lymphoid tissue destruction including lymphadenopathy, presence of multinucleated giant cells in lymphoid tissues, enlarged lymph nodes (13), and lymphocytic depletion (14), is a significant finding that might be involved in immunosuppression. Cultures of pig monocyte/macrophages are susceptible to PCV infection and support its replication, whereas PCV does not infect cultures of porcine T- or B-cells (15). Compounds that regulate differentiation of monocytes and macrophages such as PPAR-□ ligands (16) might modify the environment in which PCV replicates.

Currently, PMWS causes important economic losses to the swine industry, and vaccines against PCV2 are not available. We propose to use PMWS as a model to examine if the virally-derived immunological damage can be attenuated by supplementing diets with CLA and by increasing the levels of vitamin A in the diet. Dietary CLA or vitamin A supplementation alone is known to cause immunoenhancement. The interaction between both compounds might prevent the immunosuppression associated with PMWS by increasing the numbers and effector functions of $CD8^+$ T cells involved in antiviral activity and by regulating expression of proinflammatory cytokines in porcine monocyte/macrophages, a cell lineage critical in the pathogenesis of PMWS.

Objectives

1. To investigate the immunological changes induced during PCV2 infection involved in immunosuppression.
2. To evaluate the capacity of CLA and vitamin A to attenuate the immunological damage (i.e., depletion of T and B lymphocytes) induced by PMWS.

Procedures

Experimental design

Based on serological data collected in the Veterinary Diagnostic Laboratory at Iowa State University (ISU), Mid-western pig operations with low prevalence of PCV2 were identified. A breeding herd (Reicks View Farms, Lawler, IA) with the lowest PCV2 antibody titers as measured by ORF2 enzyme-linked immunosorbent assay (ELISA) based on a recombinant major capsid protein was chosen as a source of experimental units. Serum samples were collected from a total of 110 pregnant sows of the breeding herd 21 days prior to the estimated farrowing date. Sera were tested for the presence of ORF2-specific antibodies by indirect ELISA and the offspring from sows with the lowest S/P ratios was purchased and transported to the Large Animal Infectious Disease Isolation Facility -- a biosecurity level two containment building at ISU. This herd was

free of other viral pathogens such as porcine respiratory and reproductive virus, swine influenza virus, or porcine parvovirus. A total of 48 pigs with an initial body weight of 4.3–5.1 kg were weaned at 14 days, penned individually, fed experimental diets with free access to water, and handled according to the practices of animal care established by the Committee on Animal Care at ISU. Blocks of pigs were designed based on litter of origin, maternal S/P ratio, initial body weight, age, and gender.

Forty-eight pigs (i.e., sixteen blocks of three pigs each) were distributed into three dietary treatments: 1) soybean oil-supplemented diet and Vitamin A at NRC levels (n = 16), 2) conjugated linoleic acid (CLA)-supplemented diet and Vitamin A at NRC levels (n = 16), or 3) CLA-supplemented diet and Vitamin A at 10-fold NRC levels. Pigs were fed the experimental diets for 42 days prior to the challenge with PCV2. Prior to challenging eight of the blocks of pigs, the experimental design was a randomized complete block. Following intrainguinal lymph node challenge with PCV2 of eight of the blocks, the design became a 2×3 factorial arrangement within a split-plot design. Pigs within block were the experimental units for dietary treatment, and blocks of three pigs each were the experimental units for challenge status (i.e., PCV2-infected or non-infected).

Dietary treatments

Either a 1.33 g CLA/100 g of diet or an isocaloric and isonitrogenous soybean oil-supplemented control diet containing 1,516 μg retinyl acetate/ 1000 g of diet or a 1.33 g CLA/100 g of diet containing 15,093 μg retinyl acetate/ 1000 g of diet (Table 1) was randomly allotted to pens within blocks as previously described (2). Prior to challenge, pigs were given free access to feed for 42 d in three phases (I, 1 to 2; II, 3 to 4; and III, 5 to 6 wk). Between treatments, diets were formulated to be isocaloric and isonitrogenous to avoid energy- and/or protein-derived immunological changes (17; 18). Thus, in control diets, 2.21 g CLA source/ 100 g of diet was replaced by 2.21 g soybean oil/100 g of diet (wt/wt) to maintain both the CLA-supplemented and the control diets isocaloric within phases. Pigs were fed a CLA-supplemented & 1,516 μg retinyl acetate, a soybean oil-supplemented diet & 1,516 μg retinyl acetate, or a CLA-supplemented diet & 15,093 μg retinyl acetate during the entire experimental period. The CLA source was alkali-isomerized sunflower oil (Loders-Croklaan Lipid Nutrition, Channahon, IL). Diets were formulated to maintain or exceed current recommended nutritional requirements of the NRC (19) for pigs. Pigs, feeders and feed were weighed on a weekly basis prior and after challenge to evaluate modifications in growth [i.e., average daily gain (ADG)] and appetite [i.e., average daily feed intake (ADFI)].

Diet 3 was a positive control for the tissue and plasma vitamin A concentrations. This positive control was required because dietary CLA-supplementation increased the concentration of liver retinyl esters in rats (20). Dietary vitamin A has been shown to modulate the susceptibility of the host to viral infections. Thus, the health benefits of CLA, if any, could also be associated with the vitamin A status.

Viral challenge

Challenge inoculum consisted of one dose of type 2 porcine circovirus (PCV2) inoculated in the external inguinal lymph nodes (i.e., 1 mL each). Pigs were distributed into two separate rooms (i.e., 24 pigs per room). On d 42 of the experiment pigs in the challenged room were inoculated with PCV2 and pigs in the non-infected room were inoculated with culture media alone. Biosecurity measures were implemented to prevent contamination of the non-infected room with PCV2.

Determination of the concentration of retinyl esters and retinol in sera and liver.

Retinol and retinyl esters were measured by high performance liquid chromatography (HPLC) in sera that had been stored at -75 °C. After thawing, 150 μL aliquots of serum were diluted with 150 μL of water and deproteinated by vortexing with 300 μL of ethanol containing tocol as an internal standard and butylated hydroxytoluene (BHT) as an antioxidant. The samples were extracted twice with 1 mL of hexane; the combined supernatant was evaporated under nitrogen. The residue was dissolved in 35 μL of ethyl acetate, was diluted with 100 μL of mobile phase, mixed and ultrasonically agitated for 15 s prior to placement in the autosampler. A 15 μL volume was injected. Liver samples were partially thawed and 0.1 to 1.5 g of tissue was weighed and homogenized with 10 mL of phosphate buffered saline. The homogenate was extracted with 10 mL of hexane and centrifuged for 10 min at 2500 rpm. The upper organic layer was transferred to a 50 mL graduated tube. The aqueous layer was extracted again with 10 mL of ethyl acetate and centrifuged at 2500 rpm for 10 min. The ethyl acetate was added to the graduated tube. The sample was reextracted with 10 mL of hexane, centrifuged and the upper layer added to the pooled extracts. The aqueous phase was diluted with methanol to be 40% alcohol. This was extracted a final time with 10 mL of hexane, centrifuged and the organic layer was combined in the graduated tube. This was brought to a known volume with hexane (usually 35 mL). A 1.0 mL aliquot was dried under nitrogen and dissolved in 40 μL ethyl acetate. Then 160 μL of ethanol containing tocol as an internal standard was added and mixed. The injection volume was 15 μL.

The HPLC system consisted of a computer data system, an autosampler maintaining samples at 20 °C, a column heater at 30 °C, a programmable ultraviolet visible (ThermoSeparation Products, Fremont, CA). The separation was performed isocratically on a Spherisorb ODS2 column (3 μm, 4.6 x 150 mm) protected by a Javelin guard column containing the same stationary phase (Keystone Scientific, Bellefonte, PA). The mobile phase consisted of acetonitrile/dioxane/methanol/triethylamine (83/13/4/0.1) at a flow rate of 1.5 mL/min. The alcohol component contained 100 mM ammonium acetate. The detector was programmed to measure retinol at 325 nm for 3.0 min, then tocol at 296nm until 7.0 min then retinyl esters at 325 nm until 20 min. Linear calibration curves were prepared consisting of three concentrations of analytes which spanned the levels in the samples. The calibrants included retinol and retinyl palmitate. Other retinyl esters were calculated using the molar response factor for retinyl palmitate. Quantitation was performed by internal standard calibration using peak area ratios. In-house QC samples were analyzed at regular intervals.

Fatty acid analyses

Fatty acid analyses (triplicate sample readings from each diet) of diets was conducted using a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co, Wilmington, DE) equipped with an autosample injector and flame ionization detector (FID). A combined column, HP-225 column (7.5 m, 0.25 mm i.d., 0.25 μm nominal), an HP-wax column (15 m, 0.25 mm i.d., 0.25 μm nominal), and an HP-wax column (30 m, 0.25 mm i.d., 0.25 μm nominal) were connected using zero dead-volume column connectors (J & W Scientific, Folsom, CA), was used to improve separation. A split inlet (19:1) was used to inject samples (1 μL) into the capillary column. Ramped oven temperature conditions (180 °C for 1 min, increased to 230 °C at 2.5 °C/min, then held at 230 °C for 14 min) were used. Temperatures of both inlet and detector were 280 °C.

Helium was used as a carrier gas, and a constant column flow of 1.1 mL/min was used. Detector (FID) air, H_2, and make-up gas (He) flows were 350 mL/min, 35 mL/min, and 43 mL/min, respectively. The composition of fatty acids was calculated as percentage composition of total peak area (pA*sec). Column performance and detector response was verified using commercially available (Nu-Chek-Prep, Inc., Elysian, MN) fatty acid standards [e.g., myristic (C14:0), palmitic (C16:0), palmitoleic [C16:1 (n-9]), heptadecanoic [C17:1 (n-9)], stearic (C18:0), oleic [C18:1 (n-9)], linoleic [C18:2 (n-6)], linolenic [C18:3 (n-3)], arachidic (C20:0), arachidonic [C20:4 (n-6)], eicosapentanoic [C20:5 (n-3)], docosapentanoic [C22:5 (n-3)], docosahexanoic [C22:6 (n-3)], c9,t11; t10,c12; t9,t11 and c10,c12 CLA].

Production of recombinant capsid protein open reading frame 2 (ORF2) of PCV2

Antigen for *ex vivo* lymphocyte proliferation assays (e.g., lymphocyte blastogenesis test and PKH67 proliferation assay) was partially purified protein from Hi-Five insect cells (Invitrogen, Carlsbad, CA) infected with either wild-type baculovirus (AcMNP.wt) or recombinant baculovirus carrying the ORF2 gene of PCV2 (AcMNP.ORF2) (21) kindly provided by Dr. Prem S. Paul. At 72 hpi, recombinant baculovirus infected Hi-Five cells were transferred into a 50-mL tube and centrifuged at 800 $\times$ g for 10 min. The cell pellet was washed three times with cold PBS, and frozen at - 70°C. The infected cells were frozen and thawed three times to release viral proteins before clarification by centrifugation 800 $\times$ g for 10 min. The supernatant was diluted in PBS, laid over 6 mL of 40% sucrose in PBS, and centrifuged at 270,000 $\times$ g for 6 h using a Ti 41 SW rotor (Beckman, Palo Alto, CA). The pellet was washed three times with cold PBS before incubation with the lysis buffer for 30 min on ice. Subsequently, the viral antigen was clarified by centrifugation at 10,000 $\times$ g at 4°C for 10 min. The protein concentration was measured by using the Bio-Rad protein assay (Hercules, CA).

Indirect fluorescent antibody (IFA) assay for PCV2.

Sera samples were collected on d 0, 28, 42, 49, 56, and 63 of the experiment. The IFA assay was performed using a modification of a previously described procedure (22). For the IFA titration, serum samples were diluted at 1:10 and serial two-fold dilutions in PBS. Negative and positive control samples were diluted in PBS at 1:20 and 1:100, respectively. Positive and negative control samples as well as test serum samples were added into Immulon 2HB polystyrene microtiter plates coated with PCV2 antigen and incubated for 60 minutes at 37°C. Plates were washed 3 times with PBS. Excessive PBS was removed by tapping on absorbing paper. FITC-labeled goat anti-pig immunoglobulin G (100 μL) in PBS (1:40) was added into each well and incubated at 37°C for 30 min. Following incubation at 37°C for 30 min, plates were washed three times with PBS. Plates were read under a fluorescence microscope. Sampled with intensive nuclear staining were considered positive for the PCV2 antibody.

Recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assay (ELISA)

Sera samples were collected on d 0, 28, 42, 49, 56, and 63 of the experiment. The indirect ORF2 ELISA was performed using a modification of a previously described procedure (21). ORF2 and wild-type (WT) antigens were diluted 1:800 in the carbonate-bicarbonate coating buffer, pH 9.5. The Immulon 2HB polystyrene microtiter plates were coated with 100 μL of diluted ORF2 or WT antigen. The coated plates were

incubated at 4°C for 36 to 40 h and then stored at -20°C. The plates were then thawed and washed with PBST washing buffer (0.1 M PBS and 0.1% Tween 20). Sera were diluted 1:40 in PBS. One hundred microliters of the diluted sera were incubated with positive and negative antigen at 37°C for 30 min. Excess antibodies were removed by washing five times with PBST buffer. Plates were blotted dry between each wash and gently tapped after the last wash. Peroxidase-labeled anti-pig immunoglobulin G (100 μL) in 5% milk diluent was added into each well. Following incubation at 37°C for 30 min, plates were washed five times. 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABST) substrate (Kirkegaard & Perry Laboratories Inc.,Gaithersburg, MD) (100 μL) were added and incubated at 37°C for 15 min. The ELISA reaction was stopped by adding equal amount of 1% SDS into each well. The optical density (OD) was measured at 405 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). Data were calculated as S/P ratios.

Necropsy procedures

Pigs were anesthetized by administration of Rompun (Bayer; Shawnee, KS)/Telazol (Fort Dodge Laboratories; Fort Dodge, IA) intramuscularly and euthanized via electrocution. Forty mL of peripheral blood were collected from the subclavian vein into 50-mL conical tubes containing 5 mL of PBS with 1,000 units of heparin (Elkins-Sinn, Cherry Hill, NJ). Sections of external inguinal lymph nodes, mediastinal lymph nodes, lungs, tonsils, ileum, thymus and bone marrow were obtained, fixed in 10% buffered formalin, later embedded in paraffin, and then sectioned for histological examination. Samples of external inguinal lymph nodes were stored in PBS for lymphocyte isolation, immunophenotype and proliferation assays.

Flow cytometry

Mononuclear cells or thymocytes (i.e., 2×10^9 viable cells/L) were labeled with primary antibodies in 5×10^{-6} L of fluorescence-activated cell sorting (FACS) buffer: Phycoerythrin (PE)-labeled anti-swine-CD4 (clone 74-12-4), biotinylated IgG2a mouse anti-swine-CD8α (clone 76-2-11) (23), IgG2a mouse anti-swine-CD8β (PG164A), IgG1 mouse anti-swine-CD45 (K252.1E4, pan-leukocyte marker) (VMRD Inc, Pullman, WA), IgG1 mouse anti-swine-CD3ε (8E6), IgG2b mouse anti-swine-SWC3a (74-22-15, VMRD), or appropriate isotype control antibodies. After a 15 min incubation, cells were washed with FACS buffer and resuspended in 5×10^{-6} L volume containing the secondary antibody dilution (PE-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL), streptavidin-conjugated CyChrome (Pharmingen, San Diego, CA), PE-conjugated goat anti mouse IgG2a (Southern Biotechnology Associates Inc.). Cells were incubated for 15 min, washed twice and analyzed by flow cytometry. Flow cytometric data acquisition of peripheral blood mononuclear cells, and lymph node derived lymphocytes was performed using a FACScan (Becton Dickinson, San Jose, CA). A total of 10,000 events were saved, and data analysis on the viable cell gate (previously determined) was performed by using CellQuest software v 3.3 (Becton Dickinson). Electronic compensation was utilized to eliminate spectral overlaps between individual fluorochromes in two- and three-color flow cytometric analysis.

Proliferation assays

Complete medium was prepared by supplementing RPMI 1640 with 25mM HEPES buffer (Sigma, St. Louis, MI), 100 units/mL penicillin (Sigma), .1 mg/mL

streptomycin (Sigma), 5×10^{-5} M 2-mercaptoethanol (Sigma), 1 mM essential amino acids (Mediathech Inc.), 1 mM non-essential amino acids (Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma) and 10% FBS. Media pH were measured with a pH meter (Orion Research Inc., Beverly, MA) and adjusted to 7.4 with addition of a solution of 7.5% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA). A total of 2×10^7 PBMC from each pig (recovered using 1.077 lymphocyte separation medium, Mediatech, VA) were prepared to perform proliferation assays (Sigma) as previously described (24; 25).

Lymphocyte Blastogenesis Assay: Wells of 96-well, flat-bottomed microtiter plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) were seeded with 100 μL of whole blood and 100 μL of either media alone (non-stimulated wells), media containing the recombinant open reading frame 2 protein (rORF2) of PCV2 (20 μg/mL) or media containing concanavalin A (Con-A) (5 μg/mL) (Sigma). Preliminary validation assays using Con-A at 2.5, 5, 10, and 20 μg/mL were conducted to assess the optimal lymphocyte stimulation level to both Con A and rORF2 within our experimental conditions and genetic background of the pigs. Plates were incubated for 5 d at 37º C in 5% CO_2 humidified atmosphere. After 5 d of culture, 0.5 μCi of methyl-[^{3}H] thymidine (specific radioactivity 6.7 Ci mmol^{-1}; Amersham Life Science, Arlington Heights, IL) in 10 μL of medium was added to each well and plates incubated for an additional 20 h. Well contents were harvested onto fiber filters with a PHD cell harvester (Cambridge Technology, Cambridge, MA) and incorporated radioactivity measured by liquid scintillation counting (Packard, Meriden, CT).

PKH assay: Briefly, cells were labeled with PKH67-GL, washed and, after assessing viability using propidium iodide flourescence (Sigma), adjusted to 2×10^7 viable PBMC/mL of complete media. Cells (200,000) were added to 96-well flat bottomed microtiter plates containing 100 μL of complete medium (non-stimulated), medium plus 20 μg/mL of rORF2 of PCV2, or medium plus 5 μg/mL of concanavalin-A (Con-A). Samples from each pig were run in replicates of six for each ex vivo treatment. Cells were incubated at 37º C in 5% CO_2 humidified atmosphere for 5 d. As cells divide, PKH67-GL membrane staining diminishes resulting in a decreased mean fluorescence intensity (24). After a 5-d period, cultured cells from the six wells of the same *ex vivo* treatment and pig were pooled and prepared for immunophenotyping.

Histopathological evaluation of tissue samples

Hematoxilin-eosin (H & E)-stained lymph node, thymus, spleen, tonsil, and ileal sections were histologically evaluated on the basis of lymphoid depletion (i.e., 0) no depletion, 1) mild depletion 2) moderate depletion, and 3) severe depletion). Lung slides were evaluated on the basis of the severity of interstitial pneumonia (0 to 6) and/or bronchiolitis (0 to 3). Liver and kidney were assessed based on the severity of the lymphoplasmacytic infiltrate (0 to 3). H & E slides were labeled with accession numbers lacking any reference to either the dietary or infective treatment and were evaluated by a Board-certified pathologist. Lesion scores from all the tissues were pooled to generate an arbitrary overall lesion score for the whole animal.

Statistical Analysis

Prior to the experimental viral challenge, data were analyzed as a randomized complete block design. Post-challenge data were analyzed as a 2×3 factorial arrangement of treatments within the split-plot design. In the model, pig within block was the experimental unit for dietary treatment (subplot), and blocks of pigs within infective

status were the experimental units for infection treatment (whole plot). Analysis of variance (ANOVA) was utilized to determine the main effects of the dietary treatment (control diet, CLA-supplemented diet, or CLA & 10-fold vitamin A), the infective status (i.e., infected or non-infected with PCV2), and the interaction between dietary treatment and infective status. ANOVA was performed using the general linear model (GLM) procedure of the SAS software using the TEST statement to define the whole plot and sub-plot within the model in the program (26). A $p < 0.05$ was considered to be significant. The whole plot error (i.e., error A) being block within infective status (i.e., 14 degrees of freedom (df)) 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 dietary treatment and infective status (i.e., 28 df). The statistical model utilized was $Y_{ijk} = \mu + \text{Infection}_i + \text{error } A_{ik} + \text{Diet}_j + (\text{Infection} \times \text{Diet})_{ij} + \text{error } B_{ijk}$, μ being the general mean, Infection_i being the main effect of the i_{th} level of the challenge effect, Diet_j being the main effect of the j_{th} level of the dietary effect, $(\text{Infection} \times \text{Diet})_{ij}$ being the interaction effect between infection and diet, and errors A and B representing the random errors for the whole plot and the sub-plot, respectively.

Results

Effects of dietary treatment and infective status on growth performance.

Prior to infection with PCV2, no differences in average daily gain (ADG) or feed efficiency (G:F) were detected between dietary treatments (i.e., control, CLA-supplemented, or CLA-supplemented & Vitamin A). However, dietary CLA-supplementation caused a decrease in feed intake (ADFI) in phase, regardless of the concentration of vitamin A in the diet ($P < 0.001$). The ADFI was increased in pigs fed CLA-supplemented diets at the NRC levels of vitamin A during phase III ($P < 0.006$) (Table 3). Following challenge with PCV2, ADG was decreased in control-fed pigs from d 49 to 63 of the experiment and in pigs fed CLA-supplemented diet at 10-fold vitamin A from d 56 to 63 (Table 4). ADG in infected pigs fed CLA resembled that of non-infected pigs. No major differences in ADFI were detected following infection with PCV2.

Effects of dietary treatment and infective status on the development of lesions.

The macroscopic evaluation of tissues during the necropsy revealed that infection with PCV2 induces an enlargement of lymph nodes (i.e., external inguinal and mediastinal) ($P < 0.0001$). However no effects of dietary treatment were detected on the development of macroscopic lesions. Histopathological evaluation of H&E-stained slides demonstrated that lymphoid depletion of PCV2-infected pigs was detectable in lymph nodes, spleen, tonsils and ileum but not in the thymus (Table 5). In lymph nodes, the depletion affected primarily the lymphoid follicles (B cell regions). Although numerical differences in the lesion scores of spleen and lymph nodes were detected between dietary treatments, these differences were not statistically significant. A trend towards decreased interstitial pneumonia lesions in the lungs was observed in infected pigs fed CLA ($P < 0.14$). Overall, the lesions observed in this experiment were consistent with mild to moderate cases of PCV2-associated disease.

Antigen-specific humoral and cellular immune responses.

Although the pigs used in this experiment were selected from sows with low antibody levels against PCV2, as a result of the passive transfer of antibodies through the colostrum, maternal antibodies were detected on d 0 of the experiment which decreased with age (Figure 1). The infection-derived antibody levels were not observed by IFA until 14 days post-infection and the maximum levels were detected 21 days post-

challenge (Figure 1B). Conversely, the antigen-specific cellular immune responses (i.e., proliferation of lymphocytes following ex vivo stimulation with rORF2 of PCV2) were detectable at 7 days post-infection, peaked on d 14 post-infection (i.e., d 56 of the experiment) and declined at 21 days post-challenge (Figure 1B). Following infection, pigs fed CLA-supplemented diets containing vitamin A concentrations at the NRC recommended concentration had the greatest cellular proliferative responses [i.e., stimulation index (SI); 15.25 on d 56]. The cellular immune responses were lower in pigs fed CLA-supplemented diets containing 10-fold vitamin A (i.e., 2.28) or in pigs fed the control diets (i.e., 12.34). Thus, supplementation of diets with both CLA and vitamin A at 10-fold the NRC recommended levels down-modulate both cellular and humoral immune responses.

Both humoral and cellular responses in the non-infected group remained at the basal level (Figure 1A). Further characterization of the proliferating lymphocyte subsets using PKH67 assays revealed that most of the proliferating lymphocytes were CD4CD8 double-positive and CD8□□$^+$ T cells.

Depletion of lymphocyte subsets in peripheral blood.

In addition to evaluating the depletion of lymphocytes in tissues (Table 5), we isolated lymphocytes from whole blood and assayed for the presence of T and B cell markers. Infection with PCV2 caused a depletion of a subset of B cells co-expressing IgM and SWC3a (Figure 2B) during 14 d post-infection. On d 49 but not on d 56, the B cell depletion was attenuated by dietary CLA-supplementation. An interaction between dietary treatment and infective status was also detected ($P < 0.04$). Specifically, dietary CLA supplementation did not have any effect on the percentages of B cells in the uninfected group or prior to the infection. However, on day 49 of the experiment, in CLA-fed pigs the virally-derived B cell depletion was slightly attenuated. Even though numerically, the percentages of $CD4^+$ T cells were also decreased in infected pigs (data not shown), these changes in $CD4^+$ T cells lacked statistical significance.

Analysis of fatty acid composition of plasma.

No differences in plasma fatty acid composition were observed on day 0 (data not shown). On day 63 of the experiment, an interaction between dietary and infective treatment was observed for the percentage of stearic acid ($P < 0.02$) and a trend towards an interaction for palmitoleic acid ($P < 0.06$). Following infection with PCV2, the concentration of stearic acid in plasma was lower in infected pigs fed CLA, regardless of the dietary vitamin A concentration, and greater in infected pigs fed the control diet when compared to non-infected pigs fed the same diets. An opposite pattern was observed for the concentration of palmitoleic acid in plasma. Dietary CLA-supplementation decreased the concentration of plasma linoleic ($P < 0.03$) and arachidonic acids ($P < 0.001$) and increased the concentration of plasma cis-9, trans-11 CLA, trans-10, cis-12 CLA, regardless of the infective status.

Analysis of the vitamin A status in liver and plasma.

It had previously been suggested that dietary CLA-supplementation enhanced the vitamin A status. In this study, the CLA-supplemented diet containing vitamin A at 10-fold the NRC recommendation represented a good positive control for the vitamin A analysis. We have found that the effects of dietary CLA-supplementation on vitamin A status are very small and with a limited economical importance. Furthermore, the tight homeostatic control of the plasma concentration of vitamin A minimized the differences in plasma retinol between dietary treatments, including the 10-fold vitamin A diet (Figure

4). When fed at these high concentrations, vitamin A is primarily stored in the liver as retinyl esters (Figure 3). Infection with PCV2 favored an increase in the concentration of liver retinol in pigs fed vitamin A at 10-fold the recommended levels ($P < 0.05$).

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TABLE 2

Dietary Composition (as-fed basis)

	Control Diets[1]			
Item	**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]	—	—	—	—
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, 7-9 wk.

[2] In CLA-supplemented diets 2.21 % of CLA-60 was replaced by 2.21 % of soybean oil to maintain the diets isocaloric within phases.

[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. In CLA & Vitamin A diets, the concentration of retinyl acetate was 15,093 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).

TABLE 3

Effect of dietary treatments of pigs fed control, conjugated linoleic acid (CLA)-supplemented, or CLA and 10 fold NRC Vitamin A on growth performance prior to infection with type-2 porcine circovirus (PCV2)[1,2,3]

	Diets				*P* value[5]
Item	Control diet	CLA diet	CLA & RA	SEM	D
ADG, g					
I	326	319	313	19	0.81
II	488	538	486	25	0.08
III	756	688	739	35	0.16
ADFI, g					
I	379	378	369	18	0.86
II	654[a]	508[b]	488[b]	31	0.001
III	1,112[a]	1,229[b]	1,196[a]	34	0.006
G:F					
I	860	843	848	41	0.59
II	746	1,059	995	52	0.18
III	679	559	617	72	0.44

[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] Prior to the infection, data were analyzed as a randomized complete block design. Pig within block was considered the experimental unit. Least squares means values (n = 16) in a row for a particular growth performance criteria with different superscripts are significantly different ($P < 0.05$).

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

[5] *P*-value of the main effect of the dietary treatment [e.g., control, CLA, or CLA & retinyl acetate (RA)].

TABLE 4

Effect of dietary treatments of pigs fed control, conjugated linoleic acid (CLA)-supplemented, or CLA and 10 fold NRC Vitamin A on growth performance following infection with type-2 porcine circovirus (PCV2)[1, 2, 3]

Item	Diets	Non-infected pigs			Infected pigs[4]			SEM	*P* value[5]
		Control diet	CLA diet	CLA & Vitamin A	Control diet	CLA diet	CLA & Vitamin A		Interaction
ADG, g									
7th week		863	917	848	1034	936	798	96	0.08
8th week		892 [a]	815 [a]	818 [a]	601 [b]	848 [a]	912 [a]	84	0.03
9th week		943 [a]	952 [a]	994 [a]	752 [b]	914 [a]	528 [b]	90	0.05
ADFI, g									
7th week		1,514	1,459	1,432	1,496	1,600	1,506	51	0.31
8th week		1,624	1,556	1,614	1,712	1,886	1,851	73	0.26
9th week		2,081	1,855	1,956	1,571	1,824	1,680	110	0.11
G:F									
7th week		570 [a]	628 [a]	592 [a]	691 [a]	585 [a]	529 [b]	58	0.02
8th week		549	459	506	351	449	492	36	0.06
9th week		453 [a]	513 [a]	508 [a]	478 [a]	501 [a]	314 [b]	38	0.03

[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 a particular growth performance criteria with different superscripts are significantly different ($P < 0.05$).

[4] On day 42, eight blocks of 3 pigs each were infected as described in *Materials and Methods*. All experimentally inoculated pigs were tested for the presence of type-2 porcine circovirus in plasma.

[5] Following infection, data were analyzed as a 2×3 factorial arrangement (i.e., 2 infection status and 3 dietary treatments) within a split-plot design. Infection status represents the whole plot and dietary treatments the sub-plot. The experimental unit for the whole plot was a block of 3 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-value represents the interaction between the infection status and the dietary treatments.

TABLE 5

Effect of dietary treatment on the development of microscopic lesions following infection with type 2 porcine circovirus[1, 2, 3].

Tissue	Non-infected pigs			Infected pigs[4]			SEM	*P* value[5]		
	Control diet	CLA diet	CLA & Vitamin A	Control diet	CLA diet	CLA & Vitamin A		D	IN	D × IN
Lymph nodes	0.00 [b]	0.00 [b]	0.00 [b]	1.00 [a]	0.87 [a]	0.50 [a]	0.18	0.53	0.004	0.53
Thymus	0.00	0.00	0.00	0.00	0.00	0.00	—	—	—	—
Spleen	0.00	0.00	0.00	0.37	0.00	2.25	0.16	0.62	0.20	0.63
Tonsils	0.00[b]	0.00 [b]	0.00 [b]	0.37 [a]	0.37 [a]	0.62 [a]	0.15	0.74	0.006	0.74
Ileum	0.00	0.00	0.00	0.25	0.37	0.12	0.13	0.76	0.08	0.81
Liver	0.00	0.00	0.00	0.75 [a]	0.62 [a]	0.62 [a]	0.15	0.86	0.004	0.93
Kidney	0.00	0.00	0.00	0.25	0.12	0.12	0.10	0.75	0.14	0.86
Lungs	0.00 [c]	0.00 [c]	0.00 [c]	1.37 [a]	0.37 [b]	1.00 [a]	0.28	0.14	0.008	0.35

[1] Pigs were killed on d 63, external inguinal lymph nodes, thymus, spleen, tonsils, ileum, liver, kidney, and lungs were collected for histopathological evaluation.

[2] Least squares means values (n = 8) in a row for a particular fatty acid with different superscripts are significantly different ($P < 0.05$).

[3] Phase IV: 7-9 wk.

[4] On day 42, eight blocks of 3 pigs each were infected as described in *Materials and Methods*. All experimentally inoculated pigs were positive for PCV2 by polymerase chain reaction (PCR).

[5] *P*-value of main effects of dietary treatment (D), infection treatment (IN) and the interaction of dietary treatment by infection treatment (D × IN). Following infection, data were analyzed as a 2 × 3 factorial arrangement (i.e., 2 infection status and 3 dietary treatments) within a split-plot design. Infection status represents the whole plot and dietary treatments the sub-plot. The experimental unit for the whole plot was a block of 3 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-value represents the interaction between the infection status and the dietary treatments.

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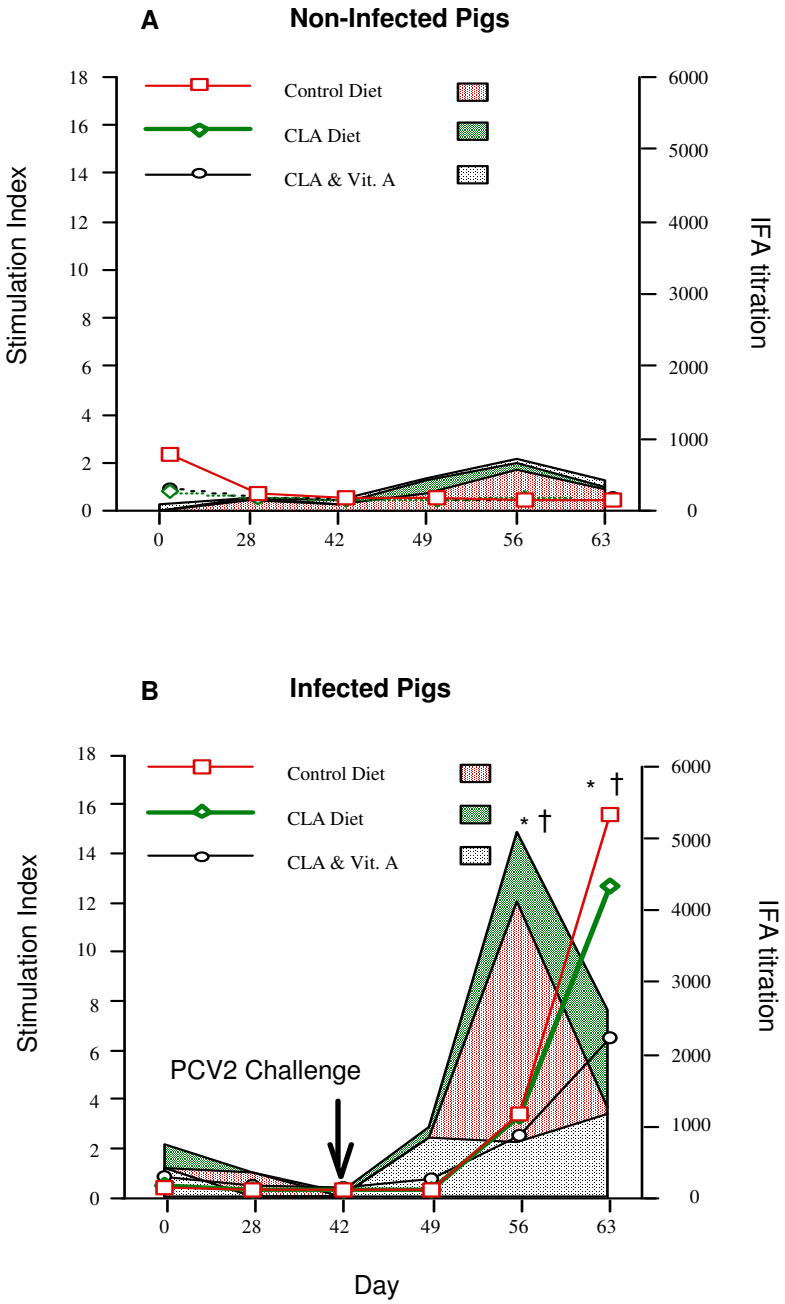

FIGURE 1. Humoral (left axis and legends) and cellular (right axis and legends) immune responses specific to porcine circovirus type 2 (PCV2) in non-infected pigs (panel A) and pigs challenged with PCV2 on day 42 of the experiment (panel B). The stimulation index was calculated by dividing the counts per minute (cpm) of antigen-stimulated wells by cpm of lymphocytes cultured with media alone. The IFA titration represents the concentration of PCV2-specific serum immunoglobulin G. Significant differences ($P < 0.05$) between treatments attributed to the main effects of infection (†), diet (∗) and the interaction between vaccine and diet (¥) are reported.

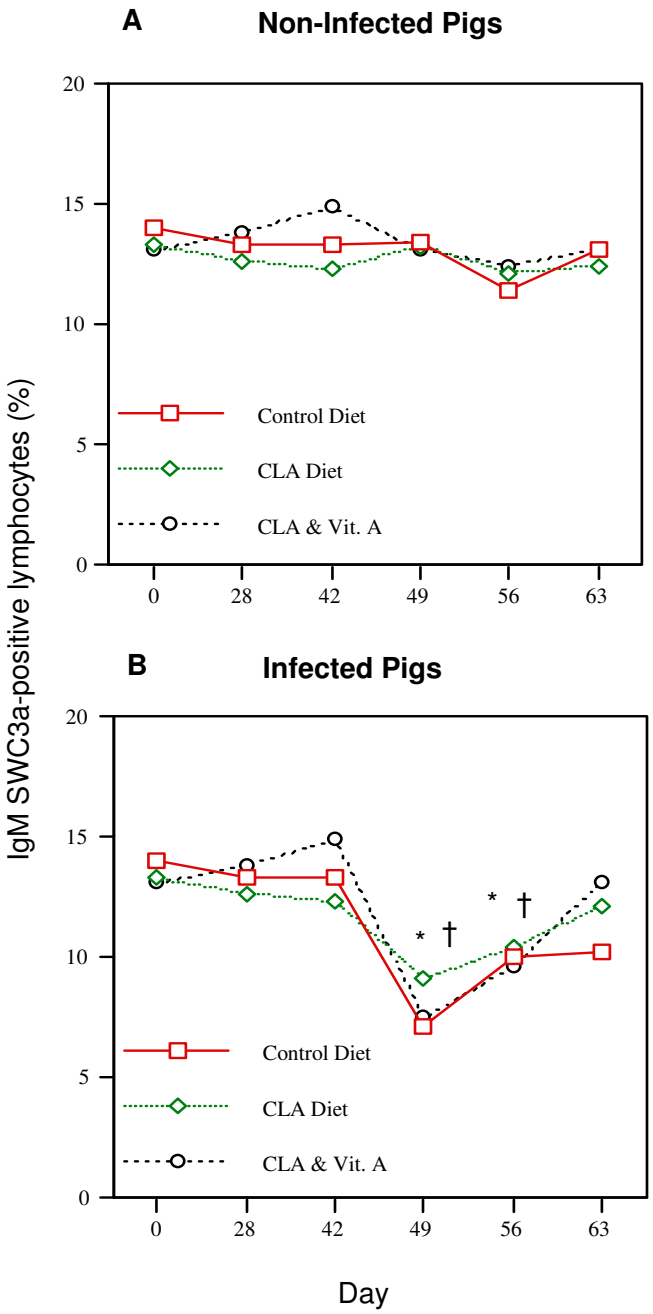

FIGURE 2. Flow cytometric analysis of $IgM^{+}SWC3a^{+}$ B lymphocytes in peripheral blood mononuclear cells recovered from non-infected (panel A) and PCV2-infected (panel B) pigs. The viral challenge occurred on day 42 of the experiment. A depletion of a B cell subset was detected during the first 15 days post-challenge. An interaction between dietary and infective treatment was detected on d 49 of the experiment ($P < 0.04$). Significant differences ($P < 0.05$) between treatments attributed to the main effects of infection (†), diet (∗) and the interaction between vaccine and diet (¥) are reported.

TABLE 6

Effect of dietary treatment on the plasma fatty acid composition following infection with type-2 porcine circovirus[1, 2, 3].

Item	Immune Modulators	Non-infected pigs			Infected pigs[4]			SEM	*P* value[5]		
		Control diet	CLA diet	CLA & Vitamin A	Control diet	CLA diet	CLA & Vitamin A		D	IN	D × IN
Myristic acid		0.75[b]	1.00[b]	1.01[b]	1.20[a]	1.20[a]	1.07[b]	0.10	0.59	0.03	0.29
Palmitic acid		17.63[b]	18.53[b]	19.74[a]	20.92[a]	19.79[a]	19.95[a]	0.57	0.58	0.01	0.10
Stearic acid		12.26[c]	14.76[b]	16.36[a]	14.28[b]	13.28[c]	13.53[c]	0.69	0.16	0.28	0.02
Palmitoleic acid		2.35[a]	1.75[b]	1.22[c]	1.53[b]	3.01[a]	2.40[a]	0.35	0.45	0.16	0.06
Vaccenic acid		1.63	1.83	1.61	2.30	1.62	1.85	0.23	0.65	0.34	0.34
Oleic acid		18.56	18.58	19.99	19.43	18.56	19.24	0.42	0.15	0.94	0.31
Linoleic acid		32.00[a]	26.25[b]	26.35[b]	28.04[a]	26.38[b]	26.63[b]	1.21	0.03	0.34	0.29
c9, t11 CLA		0.00[b]	4.81[a]	4.34[a]	0.00[b]	5.04[a]	4.79[a]	0.67	0.001	0.69	0.61
t10, c12 CLA		0.00[b]	3.06[a]	2.56[a]	0.00[b]	3.46[a]	3.61[a]	0.46	0.001	0.46	0.88
Other CLA isomers		0.00[b]	0.68[a]	0.67[a]	0.00[b]	0.74[a]	0.72[a]	0.19	0.05	0.12	0.55
Linolenic acid		1.51	2.17	0.78	1.39	1.58	0.91	0.52	0.28	0.71	0.84
Arachidonic acid		1.53[a]	0.86[b]	0.67[b]	1.46[a]	0.85[b]	0.63[b]	0.76	0.001	0.23	0.09

[1] Pigs were killed on d 63, whole blood was collected, plasma was isolated and the fatty acid composition of the plasma was analyzed by gas chromatography.

[2] Least squares means values (n = 8) in a row for a particular fatty acid with different superscripts are significantly different ($P < 0.05$).

[3] Phase IV: 7-9 wk.

[4] On day 42, eight blocks of 3 pigs each were infected as described in *Materials and Methods*.

[5] *P*-value of main effects of dietary treatment (D), infection treatment (IN) and the interaction of dietary treatment by infection treatment (D × IN). Following infection, data were analyzed as a 2 × 3 factorial arrangement (i.e., 2 infection status and 3 dietary treatments) within a split-plot design. Infection status represents the whole plot and dietary treatments the sub-plot. The experimental unit for the whole plot was a block of 3 littermate pigs and the experimental unit for the sub-plot was pig within a block. The P-value represents the interaction between the infection status and the dietary treatments.

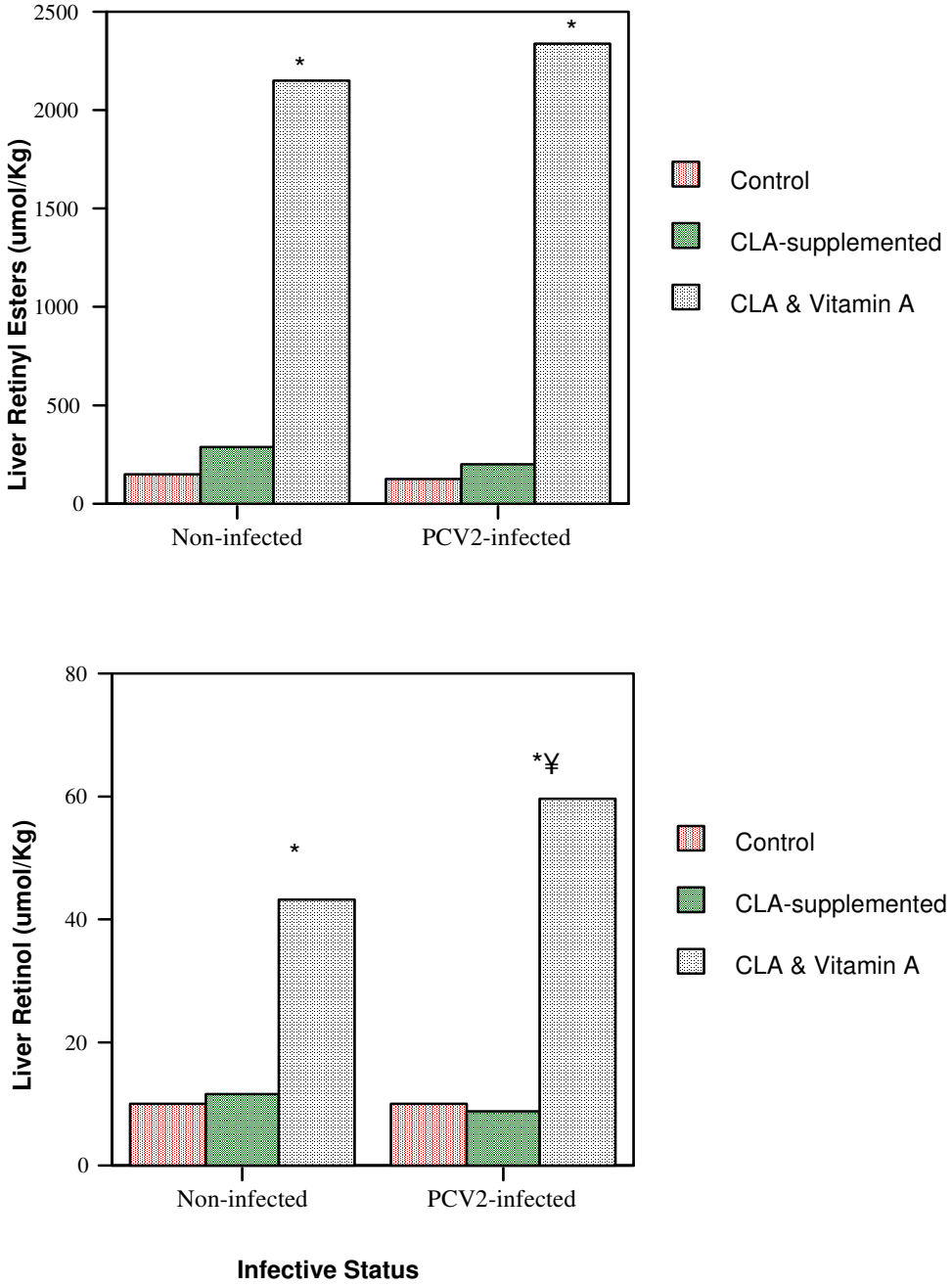

FIGURE 3. Accumulation of retinyl esters (upper panel) and retinol (bottom panel) in liver after feeding the experimental diets for 63 days. Supplementation of diets with vitamin A at 10-fold the NRC recommended level resulted in increased liver concentrations of retinol and retinyl esters. Significant differences ($P < 0.05$) between treatments attributed to the main effects of infection (†), diet (∗) and the interaction between vaccine and diet (¥) are reported.

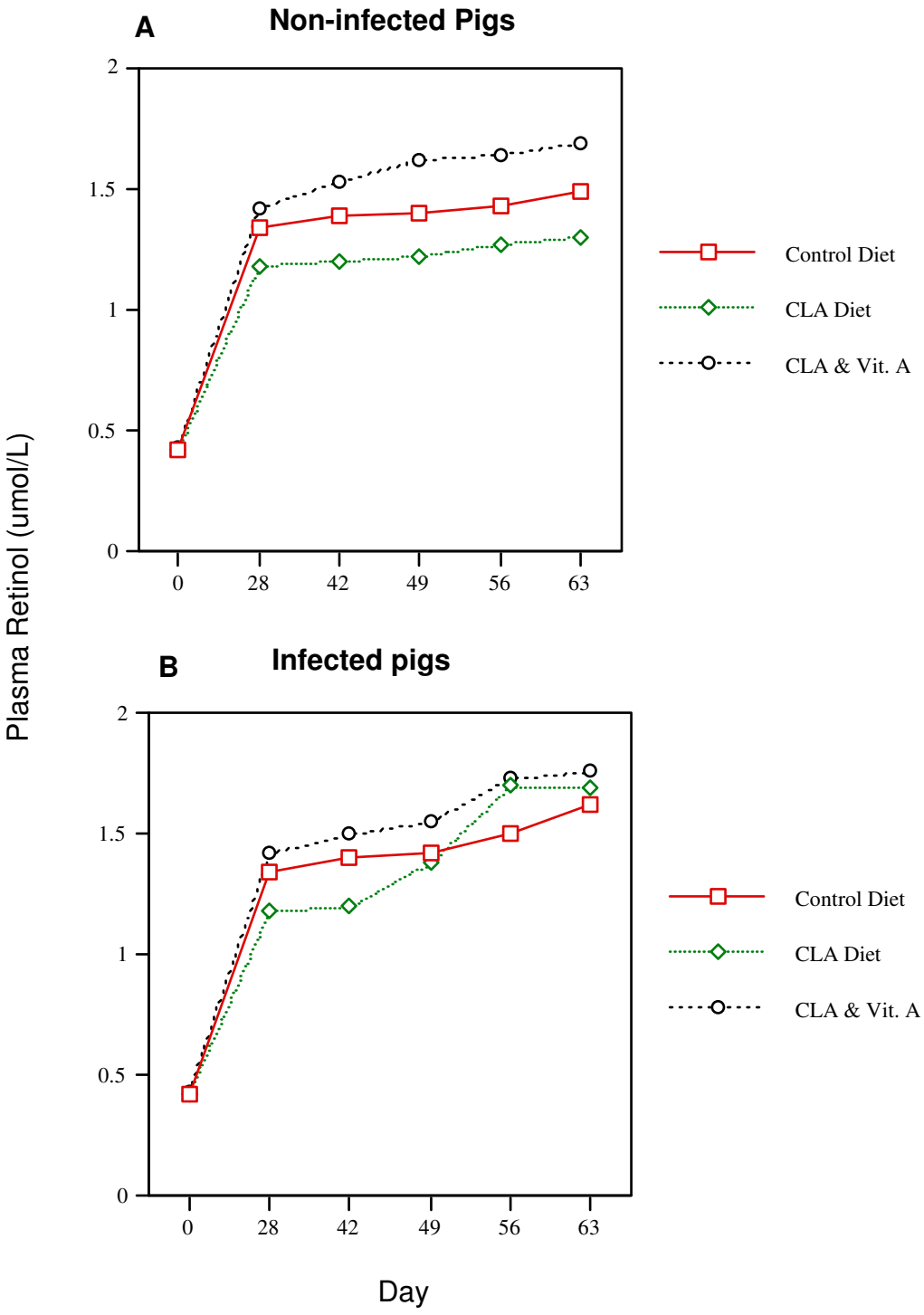

FIGURE 4. Plasma retinol concentrations in infected (panel B) and non-infected (panel A) pigs. No statistically significant differences were detected.