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Original Article

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Abstract

This study evaluated the effect of oxidized soybean oil on the immune response to porcine reproductive and respiratory syndrome modified live vaccine (PRRS-MLV), and the oxidative stress status in nursery pigs. Seventy castrated weaned pigs, from a free PRRS virus infected herd, were divided into two groups: treatment group (n=63) and control group (n=7). The treatment pigs were vaccinated with the PRRS-MLV and then divided into three groups based on different diet types containing different soybean oils: (A) 5% fresh oil, (B) 5% heated oil for 43 h, and (C) 5% heated oil for 38 h. The control pigs were not vaccinated and consumed (A) diet. Blood samples were collected for PRRS immune response and for measuring oxidative status. No significant differences were observed among groups in malondialdehyde (MDA), superoxide dismutase-1 (SOD-1), ELISA titer, and SN antibodies against PRRS virus. Notably, the percentage of interferon-gamma (IFN-γ) producing cell increased significantly in group A in comparison to that in the other groups on D28 and D56. In contrast, the percentage of interleukin-10 (IL-10) producing cell was highest in group C, followed by group B and group A, respectively. Moreover, group C exhibited a higher number of IL-10 producing cells compared to group A on D56 (p=0.082). Oxidized soybean oil exerts a negative effect on the cell-mediated immune response to PRRS-MLV, especially in pigs fed oil containing high peroxide value and high numbers of total polar compounds. These findings suggest that pig farmers should be more concerned with the quality of oil used in the diet for nursery pigs.

Keywords: Immune Response, Oxidative stress, Oxidized Soybean Oil, Nursery Pigs, PRRSV

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Introduction

Inefficiency and inconsistency in pig rearing are common problems encountered in pig farms throughout the world, including Thailand. There are several factors influencing this problem, such as swine diseases, especially Porcine Reproductive and Respiratory Syndrome (PRRS), farm management, numbers and quality of farm laborers, the market price of pork along with the price and quality of the raw materials; all of these factors affect production efficiency in the pig industry. This is especially true during a decline in the market price of the finished pigs and rising feed prices, which are the reasons for the use of lower-grade raw materials in order to reduce the costs of production since the feed cost is approximately 65%–70% of the total cost for pig production. Oxidized oil is frequently substituted for the high-grade oil in pig diet. Even though it is known that the oxidized oil may have an impact on the production efficiency and the health of the pigs, it is nonetheless used widely in Thai commercial pig farms and feed mills (Boonsoongnern *et al*., 2017).

Oxidative stress is a condition of imbalance between free radical generation and antioxidant levels in the body. A good oil is altered in its physical and chemical composition when exposed to high temperatures and moisture. In addition, oxidized oils contain several toxic compounds, including 1,4-dioxane, benzene, toluene and hexyl-benzene. Several studies conducted on rats and mice have indicated that oxidized oil is able to induce oxidative stress and exert a negative impact on growth performances (Olivero David *et al*., 2010; Olivero *et al*., 2011; Varady *et al*., 2011; Yen *et al*., 2010).

Oxidized oil or lipid peroxidation is a complex and dynamic process that degrades and produces numerous peroxidation compounds that can attack an oxygen molecule in unsaturated fatty acids (Labuza and Dugan, 1971). The rate of oxygen uptake by a fatty acid correlates with the degree of unsaturation and an increasing of the carbon chain length (Naudi *et al*., 2011). Soybean oil contains a high number of unsaturated fatty acids, so it is easily induced to become oxidized oil (Perkins, 1995).

PRRS virus (PRRSV) affects the reproductive and respiratory systems, resulting in late-term abortion, mummification, stillbirth, coughing, abdominal breathing, cyanosis, and death. It has been reported that the virus suppresses the immune response in infected pigs, as evidenced by the outcomes of significantly increased interleukin 10 (IL-10) production by the peripheral blood mononuclear cells (PBMCs) (Couper *et al*., 2008; Diaz *et al*., 2005; Suradhat and Thanawongnuwech, 2003). In the case of a chronic PRRSV infection, the virus has been detected in several organs, especially in the lymph nodes, lungs, and tonsils, which can be evidence of low serum neutralization (SN) antibody titers and decreased interferon-gamma (IFN-γ) producing PBMCs (Diaz *et al*., 2005; Lopez and Osorio, 2004; Suradhat *et al*., 2015). In this context, the present study aimed to determine the effects of oxidized soybean oil on the immune response to PRRS modified live vaccine and to evaluate the oxidative stress parameters in nursery pigs.

Materials and Methods

***Animals and experimental design*:** The procedure for animal use employed in the present study was approved by the Institutional Animal Care and Use Committee, Kasetsart University. The harvest was conducted under the Thai Research Institution No. ACKU 04260. A completely randomized design with sub-sampling was employed for the study. The experiments were performed in the Veterinary Demonstration Farm Unit, Kasetsart University, Kampheang Saen Campus, Nakhon Pathom province, Thailand. The nursery building, with each pen of size 2.0 × 2.0 meters (wide × length), was provided for the weaning pigs. Each pen had one water nipple and one mechanical feeder. All the pigs were labeled with individual identity numbers. The duration of the study was eight weeks.

Seventy 25-day-old castrated pigs (Landrace × Large White; BW 8.66 ±1.42 kg) were obtained from a free PRRSV-infected herd. The pigs were divided into two groups: the treatment group (n = 63) and the negative control group (n = 7). The pigs in the negative control group were housed in a different barn to separate them from the pigs in the treatment group. The negative control group pigs did not receive vaccination with the PRRSV modified live vaccine (Ingelvac PRRS® MLV, Boehringer Ingelheim, Germany) and consumed a diet containing 5% fresh soybean oil. The treatment group pigs received vaccination with the PRRSV modified live vaccine, following which they were sub-divided into three sub-groups (n = 21 for each sub-group): (A) pigs that were fed on a diet containing fresh soybean oil, (B) pigs fed on a diet containing soybean oil heated for 43 h, and (C) pigs fed on a diet containing soybean oil heated for 38 h (Table 1). There were seven pens in each sub-group, i.e., three pigs per pen.

Table 1 Experimental design

Groups (pig)	PV (mEq/kg of oil)	TPC (%)	PRRS vaccination
Negative control (7)	0.88	4.50	No
A (21)	0.88	4.50	Yes
B (21)	98.00	> 40.00	Yes
C (21)	168.00	37.00	Yes

PV, peroxide value; TPC, total polar compounds; PRRS, porcine reproductive and respiratory syndrome

Preparation of oxidized soybean oil and diets: The oxidized soybean oil was prepared by heating fresh soybean oil to 110–120 °C, while continuously injecting oxygen at a flow rate of 10 L/min for different total time durations (38 h and 43 h). The peroxide value (PV) and the percentage total polar compound (%TPC) of

the prepared oils were measured following the methods described in previous studies (Boonsoongnern *et al*., 2017). The fresh soybean oil was observed to contain 0.88 mEq/kg of PV and 4.5% TPC.

The PV for the oxidized soybean oils were 168 and 98 mEq/kg of PV, and the %TPC in the oxidized soybean oils were 37% and >40% (for 38 h and 43 h, respectively). The oils were stored at 4 °C until use. In order to prepare the diets, broken rice-soybean-based diet was mixed with 5% of oil, generating the final experimental diets. Feed formulations were isocaloric diets, including the phase 1 diet (Day 0–28) and phase 2 diet (Day 29–56), as presented in Table 2.

Table 2 Experimental feed composition and determined parameters

Items	Day 0-28	Day 29-56
Ingredients,%		
- Broken rice	43.33	52.42
- Soybean	9.62	19.06
- Soy protein concentrate (Milpro®200)	9.62	4.76
- Full fat soy	14.44	14.29
- Milk replacer (Agrolac®20/40)	14.44	-
- Fresh or oxidized soybean oil	5.00	5.00
- Mono-dicalcium phosphate P21	1.44	2.29
- Lame stone	0.96	0.95
- Salt	0.19	0.48
- L-lysine HCl	0.39	0.35
- DL-methionine	0.29	0.21
- L-threonine	0.19	0.17
- Vitamin and mineral premix[1]	0.24	0.24
Calculated analysis		
- ME, kcal/kg	3,746.02	3,495.24
- CP,%	21.44	20.72
- Crude fiber,%	2.33	2.81
- Crude fat,%	11.06	8.21
- Calcium,%	0.91	0.85
- Available P,%	0.40	0.45
- Total lysine,%	1.51	1.38
- Met+Cyst:Lys	60.91	60.87
- Thr:Lys	65.56	66.67
- Try:Lys	18.54	19.57

[1] One kilogram of vitamin and mineral premix contains vitamin A, 4,000,000 IU; vitamin D3, 400,000 IU; vitamin E, 13,000 g; vitamin K3, 0.6 g; vitamin B1, 0.8 g; vitamin B3, 1.3 g; vitamin B6, 0.8 g; vitamin B12, 0.8 g; niacin, 9,000 g; pantothenic acid, 6,000 g; biotin, 40 g; folic acid, 0.3 g; choline, 35,000 g; Cu, 32,000 g; Fe, 36,000 g; Mn, 10,000 g; Zn, 30,000 g; Co, 0.05 g; Se, 0.06 g and I, 0.4 g

Sample collection: Blood samples of the animals were obtained by extracting blood from the external jugular vein of the pigs at Day 0, 14, 28, and 56. The serum and plasma samples were stored at −20 °C until to be used for analysis. The sera were used for evaluating the antibodies against PRRSV using enzyme-linked immunosorbent assay (ELISA) and serum neutralizing (SN) techniques. Moreover, the heparinized blood at Day 0, 28, and 56 was used for evaluating the PRRS-specific IFN-γ and the IL-10 producing PBMCs, and for analyzing the enzymatic antioxidant activity and lipid peroxidation.

Laboratory analysis

Analysis of antibody response against PRRSV using ELISA technique: All the serum samples obtained at Day 0, 14, 28, and 56 were analyzed for antibodies against PRRSV using the ELISA IDEXX PRRS 3X Ab Test Kit (IDEXX Laboratories, Inc., USA). The analysis protocol was performed in accordance with the manufacturer's instructions. S/P ratio greater than 0.4 was considered a positive detection of the antibodies against PRRSV.

***Analysis of antibody response against PRRSV using Serum neutralizing technique*:** PRRSV was isolated from the Thai pig farm in 2004 (accession number MK774669) and propagated in MARC-145 cells. The SN test was performed for all the sera obtained on Day 0, 28, and 56. The serum samples were subjected to heat treatment at 56 °C for 30 min, followed by two-fold dilution in DMEM (Merck KGaA, Darmstadt, Germany) supplemented with 10% FBS (Merck KGaA) and 50 µg gentamicin (Thermo Fisher Scientific, USA). The dilution of the serum sample was performed in a 96-well plate, and an equal volume of the virus solution was added to each well with a titer of 10^2 $TCID_{50}$/mL. The microplate was then incubated at 37 °C for 60 min. The serum-virus mixtures were transferred to a fresh 96-well plate containing a monolayer of MARC-145 cells. The SN titer was defined as the highest dilution of the serum that neutralized the cytopathic effects (CPE) on Day 3 post-incubation. Back titration of the virus was performed each time in order to confirm the correctness of the viral titration. Each sample was run in duplicate and the mean of the titers was calculated as the final value (Yoon *et al*., 1994).

Flow cytometry: PRRS-specific IFN-γ and IL-10-producing PBMCs were analyzed using the flow cytometry technique. The PBMCs at Day 0, 28, and 56 were isolated using Ficoll®-Plaque Premium density gradient media (Merck KGaA, Darmstadt, Germany), following the method described in a previous study (Wongyanin *et al*., 2010). In order to conduct the in vitro stimulation, 1×10^6 PBMCs were cultured in a 96-

well-plate, with 0.05 multiplicity of infection (m.o.i.) of the PRRSV or mock, for 48 h. At 42 h of the incubation period, a protein transport inhibitor (GolgiStop™; BD Biosciences, San Jose, CA, USA) was added to the culture for accumulating IFN-γ and IL-10 inside the cells. At the end of the incubation period, the stimulated cells were transferred to a U-shape-96-well plate and washed twice using 1X phosphate-buffered saline (PBS) [pH 7.4]. In order to perform PBMC-IFN-γ staining, the PBMCs were stained intracellularly in several steps, for the detection of cytokines present in these cells. In the first step, the cells were permeabilized and fixed with 100 µL of BD Fix and Perm™ (BD Biosciences) at 4 °C for 20 mins in the dark. In the second step, the cells were washed twice with 250 µL BD Perm/Wash™ buffer (BD Biosciences) and stained with 1:100 of anti-IFN-γ-Alexa 647 (BD Biosciences). In the third step, after two washes, the stained cells were resuspended in 200 µL of 2% paraformaldehyde prepared in PBS in order to fix and preserve the cells until used for the analysis of the percentage of IFN-γ-producing cells using a flow cytometer (BD Accuri™ C6 Cytometer, BD Biosciences).

In order to perform the PBMC-IL-10 staining, the cells were stained with 0.5 µg/mL of biotinylated anti-IL-10 (Invitrogen, USA) at 4 °C for 30 min in the dark, followed by washing of the cells twice with 250 µL BD Perm/Wash™ buffer. Subsequently, the cells were secondarily stained with 1:100 Streptavidin-PE (BD Biosciences) at 4 °C for 30 min in the dark, followed by two washes with 250 µL BD Perm/Wash™ buffer. Lastly, the stained cells were resuspended in 200 µL of 2% paraformaldehyde prepared in PBS in order to fix and preserve the cells until they were used for the analysis of the IL-10-producing cells using the flow cytometer.

The background cut-off and flow cytometer validation were conducted by performing IgG1-PE isotype control staining using six and eight peak bead runs (BD Biosciences), respectively. Acquisition of 30,000 events and analyses were performed using the Accuri C6 flow cytometer (BD Bioscience). The data provided the mean percentage (±standard deviation; SD) of the cytokine-producing cells, which were calculated from the following expression: [(percentage of cytokine-producing cells obtained from the PRRSV) – (cultured PBMC − percentage of cytokine-producing cells obtained from the mock-cultured PBMC)].

Oxidative stress parameters: Cu/Zn-superoxide dismutase (SOD-1) and thiobarbituric acid reactive substances (TBARS) are commonly used as markers of oxidative stress. TBARS, which are among the most crucial chemical substances of lipid peroxidation, were quantified for measuring the malondialdehyde (MDA) levels. The plasma samples (Day 0, 28, and 56) were analyzed for the levels of enzymatic antioxidant activity using the ELISA assay kits (Cayman Chemical Company, USA). The end product of SOD-1 and TBARS were evaluated by measuring the OD at 450 nm and 530 nm, respectively.

Statistical analysis: All the results were analyzed using one-way analysis of variance (ANOVA) with repeated measures and compared using Tukey's multiple comparison test, utilizing the R program version 3.1.3, R Core Team (2014), R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria (URL http://www.R-project.org/). The comparison between the groups or the data were considered to be statistically significant when $p < 0.05$.

Results

Immune response: S/P ratios of the antibodies against PRRSV exhibited an increasing trend from D0 to D28, followed by a slight decrease at D56. All pigs exhibited positive ELISA results (cut-off S/P ratio ≥ 0.4) at two weeks after the PRRS vaccination. No significant differences were observed in the antibody response against PRRSV among the different groups (Fig. 1A). All the pigs vaccinated with PRRS modified live vaccine exhibited continuously increasing S/P ratios of the antibodies against PRRSV from D0 until D28, and a slight decrease in the ratio at D56. According to the results of the SN titer, continuously increasing titers were observed until D56 post-vaccination, although there were no significant differences between the groups. However, the SN titer exhibited the lowest value at D28 and D56 in group C (Fig. 1B). The SN antibody against PRRSV demonstrated results similar to those obtained in ELISA. Although the pigs vaccinated with the PRRS vaccine were fed on diets containing the highest PV (group C), they exhibited low levels of SN titer against PRRSV compared to the other groups (groups A and B).

The IFN-γ was produced by the cells at D28 post-vaccination, and its levels were increased sharply from D0 to D56, especially in group A. Notably, the pigs belonging to group A exhibited a significantly higher number of IFN-γ-producing cells at D28 and D56, compared to those in group B and C (Fig. 1C). On the other hand, the highest numbers of IL-10-producing cells at D28 and 56 were obtained for group C, followed by B and A, respectively. As expected, the percentage of the IL-10-producing cells at D56 obtained for group C was higher ($p = 0.082$) than that obtained for group A (Fig. 1D). The pigs fed on the oxidized oils containing the highest level of PV exhibited a high percentage of IL-10-producing cells, especially at D56, compared to the other groups.

Oxidant and antioxidant: The results of the present study demonstrated that the levels of MDA and SOD-1 exhibited an increase from D0 to D56 in all the groups, although no significant differences were observed between the groups. However, the concentration of plasma MDA at D56 was observed to be the highest in group C, followed by group B and group A, respectively (Table 3)

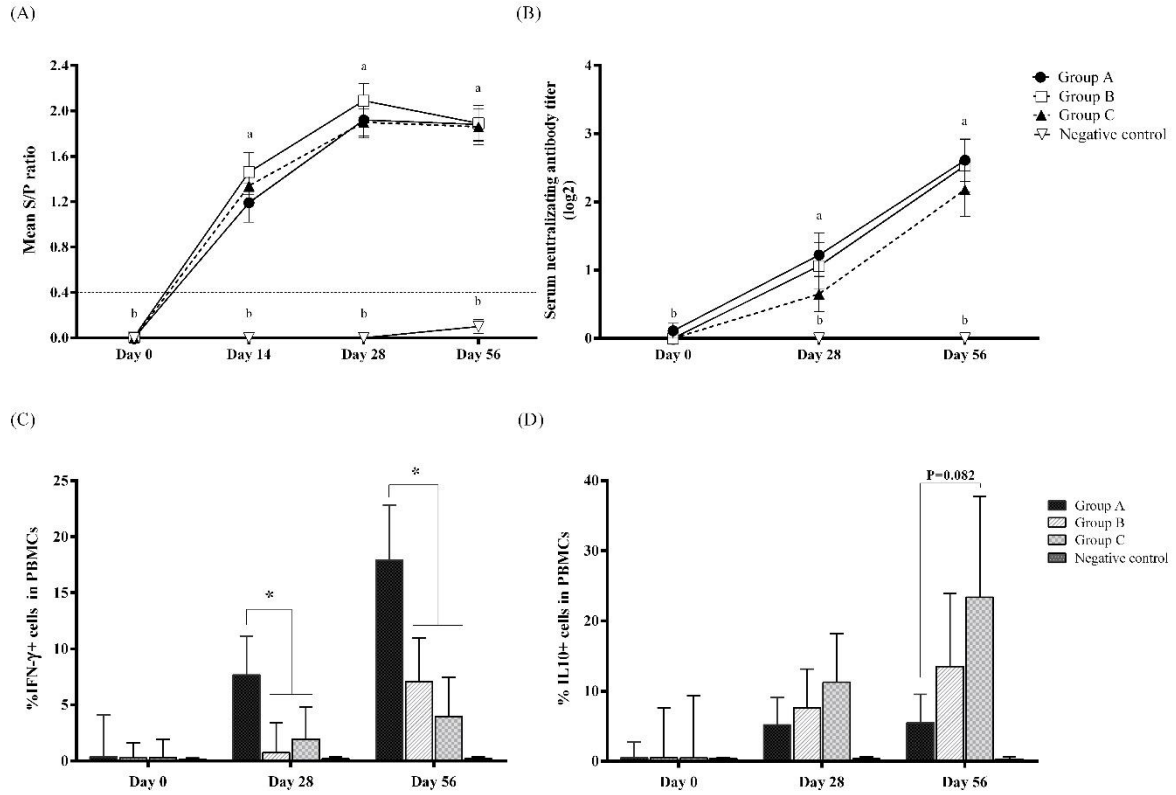

Figure 1 The PRRS immune response of nursery pigs fed on the different level of oxidized soybean oils. (A) ELISA response, (B) serum neutralizing antibody against PRRSV, (C) PRRS-specific IFN-γ produced by the PBMCs, (C) PRRS-specific IL-10 produced by the PBMCs. Asterisks and different letters indicate significant differences among the groups at the same time point (mean ±SD). Statistical analysis was performed using the one-way ANOVA with repeated measures and Tukey's correction for multiple comparisons, * $p < 0.05$.

Table 3 The levels of lipid peroxidant and antioxidant in the blood plasma of nursery pigs

Group (n)	MDA (mM/L)			SOD-1 activity (U/L)		
	D 0	D 28	D 56	D 0	D 28	D 56
A (14)	2.46 ±0.17^{c}	8.03 ±0.95^{b}	36.74 ±7.18^{a}	1.43 ±1.0^{c}	15 ±97 ±3.78^{b}	84.94 ±5.92^{a}
B (13)	2.23 ±0.15^{c}	8.57 ±1.52^{b}	58 ±93 ±9.92^{a}	1.40 ±1.1^{c}	13.95 ±2.81^{b}	87.67 ±10.63^{a}
C (14)	2.07 ±0.14^{c}	7.15 ±0.86^{b}	68.99 ±10.23^{a}	7.42 ±3.00^{c}	20.83 ±4.07^{b}	85.87 ±7.30^{a}
Significant	ns	ns	ns	ns	ns	ns

Results are presented as mean ±SD
MDA, malondialdehyde; SOD, superoxide dismutase
ns = the mean values do not differ significantly ($p > 0.05$)
$^{a, b, c}$ mean values in a row with different superscript letters were significantly different ($p < 0.05$)

Discussion

This study showed similar results to the previous reports that the ELISA antibody response against PRRSV could be detected beginning from the period between Day 9 and 12 after the PRRSV infection or vaccination with the attenuated PRRSV live vaccines (Lopez and Osorio, 2004). The study finding was in agreement with those in previous studies that reported gradual and low-level responses of SN titer after vaccination with modified live PRRS vaccine (Lopez and Osorio, 2004; Osorio *et al*., 1998).

The number of IFN-γ-producing cells correlated with the PRRSV protective immunity through which the virus was eliminated using the cellular immunity provided by the PRRSV-specific IFN-γ-producing cells (Charerntantanakul *et al*., 2006; Loving *et al*., 2015; Martelli *et al*., 2013). It is noteworthy that the oxidized oils exerted a negative impact on immunomodulatory activity (Brody, 2016).

IL-10 is a well-known anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines. IL-10 is known to impede the activity of the Th1 cells, NK cells, and macrophages, for the optimal clearance of pathogen, which may lead to tissue damage (Couper *et al*., 2008). In addition, IL-10 may act directly on the $CD4^{+}$ T cells in inhibiting the proliferation and production of IL-2, IFN-γ, IL-4, IL-5,

and TNF-α (Couper *et al*., 2008; Joss *et al*., 2000; Moore *et al*., 2001; Schandené *et al*., 1994). Moreover, Suradhat and Thanawongnuwech (2003) observed that the genotypes of both the PRRSV strains, namely the North American (NA) and European (EU) strains, significantly induced IL-10 production through PBMCs. The findings of the present study, therefore, indicate that the oxidized oils impaired the immune responses. The results of the present study are in agreement with the findings of previous studies conducted on poultry and swine (Calder, 2008; Dibner *et al*., 1996; Leff, 2001; Liang *et al*., 2015a). Importantly, MDA is produced during the lipid peroxidation process. It is more stable and changes many cell membranes especially immune cells *(*Van der Paal *et al*., 2015). Low levels of MDA and MDA adducts can be immune complement inducers. In the opposite way, high levels of them showed an adverse effect on immune cells (Willis *et al*., 2004).

Oxidative stress is induced by the common stressors in nursery pigs, such as housing management, dietary factors and disease susceptibility on pig farms (Forbes, 2007; Jones *et al*., 2001; Melin *et al*., 2004; Moeser *et al*., 2007). Oxidative stress may be evaluated through multiple markers associated with the overproduction of the reactive oxygen species (ROS). Lipid peroxidation is commonly used as an indicator of the ROS-mediated damage to cell membranes. MDA is useful as lipid oxidative stress marker, while SOD-1 represents an antioxidant enzyme. The MDA and SOD-1 levels exhibited a sharp increase from the beginning to the end of the study. Meanwhile, the levels of lipid peroxidants and antioxidants did not differ between the sub-groups when measured on the same day (D0, D28, and D56). Rosero and colleagues (Rosero *et al*., 2015) also reported that nursery pigs fed on oxidized soybean oil exhibited high levels of MDA concentration in jejunal mucosa. Some researchers found that oxidized soybean oil increased the concentration of MDA in the jejunum and liver in broilers, indicating lipid peroxidation in these tissues (Liang *et al*., 2015b; Tan *et al*., 2018; Zhang *et al*., 2011). This finding was in contrast to a previous study conducted by Petrovic and colleagues, who reported a significant decrease in the levels of MDA and SOD after weaning (Petrovic *et al*., 2008).

In conclusion, it was observed that the oxidized soybean oil induced negative effects on the PRRS cell-mediated immune response, especially in pigs fed on diets containing high PV and % TPC. This finding has consequences for pig farms that are encountering several diseases or stressors. It is strongly recommended that pig farmers have concern for and select only good quality oil, which can be identified by utilizing the PV and %TPC parameters for preparing pig diets, as healthy pigs will provide better farm performances and farm benefits.

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