NON RUMINANT NUTRITION

Dietary soy isoflavones reduce pathogen-related mortality in growing pigs under porcine reproductive and respiratory syndrome viral challenge

Brooke N. Smith,^{*} Maci L. Oelschlager,^{*}
Muhammed Shameer Abdul Rasheed,^{*} and Ryan N. Dilger^{*,†,1}

^{*}Department of Animal Sciences, University of Illinois, Urbana, IL, [†]Division of Nutritional Sciences, University of Illinois, Urbana, IL

¹Corresponding author: rdilger2@illinois.edu

ORCID number: 0000-0003-2538-2845 (R. N. Dilger).

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important disease, and ingestion of soy isoflavones (ISF) may benefit PRRSV-infected pigs due to demonstrated anti-inflammatory and antiviral properties. The objective of this experiment was to recreate immunological effects previously observed in young pigs infected with PRRSV receiving ISF and determine how those effects influence growth performance during the entire growth period from weaning to market. In total, 96 weaned barrows were group housed in a biosafety level-2 containment facility and allotted to 1 of 3 experimental treatments that were maintained throughout the study: noninfected pigs received an ISF-devoid control diet (NEG, $n = 24$), and infected pigs received either the control diet (POS, $n = 36$) or that supplemented with total ISF in excess of 1,600 mg/kg (ISF, $n = 36$). Following a 7-d adaptation, weanling pigs were inoculated intranasally with either a sham-control (PBS) or live PRRSV (1×10^5 TCID₅₀/mL, strain NADC20). After inoculation, individual blood samples ($n = 8$ to 12/treatment) were routinely collected to monitor viral clearance and hematological parameters, including serum neutralizing anti-PRRSV antibody production. Pen-based oral fluids were used to monitor PRRSV clearance at later growth stages. A 1- or 2-way ANOVA was performed to compare experimental treatments depending on whether the outcome was repeatedly measured. In general, PRRSV infection decreased performance during early growth phases, resulting in 5.4% lower final BW for POS vs. NEG pigs ($P < 0.05$). Dietary ISF elicited inconsistent effects on growth performance, increased ($P < 0.05$) neutrophil cell counts and the relative proportion of memory T-cells, and decreased ($P < 0.05$) the time to full PRRSV clearance from oral fluids. Dietary ISF also elicited earlier, more robust anti-PRRSV neutralizing antibody production when compared with POS pigs. Additionally, and most notably, POS pigs experienced ~50% greater infection-related mortality rate vs. ISF pigs ($P < 0.05$), which may have significant economic implications for producers. Overall, dietary ISF ingestion supported immune responses and reduced mortality in PRRSV-infected pigs when fed to growing pigs though the biological mechanism of these effects remains unclear.

Key words: disease, immune system, isoflavones, porcine reproductive and respiratory syndrome virus, soybean, swine

Abbreviations

CD3/4/8+	cluster of differentiation 3/4/8 positive
Ct	cycle threshold
DDGS	dried distillers grains with soluble
DPI	day post-inoculation
E2	17-beta-estradiol
ER-β	estrogen receptor beta
IFN-α	interferon alpha
IFN-β	interferon beta
ISF	isoflavones/PRRSV-positive experimental treatment
ISS-VRF	Innovative Swine Solutions Veterinary Research Facility
NEG	PRRSV-negative control treatment
NRC	National Research Council
PBMC	peripheral blood mononuclear cells
POS	PRRSV-positive control treatment
PRRSV	porcine reproductive and respiratory syndrome virus
RBC	red blood cell(s)
TNF-α	tumor necrosis factor-alpha
USDA-ARS	United States Department of Agriculture Agricultural Research Service
WBC	white blood cell(s)

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be one of the leading pathogens causing high economic impact in modern swine production (Holtkamp et al., 2013). Due to its prevalence and lack of highly effective vaccine options (Loving et al., 2015), nutritional intervention or management strategies are of high interest for PRRSV mitigation due to relatively low cost and ease of implementation. It has been demonstrated that increasing the level of soybean meal in diets fed to pigs under immune stress reduces adverse effects of illness and may improve growth performance (Boyd et al., 2010; Rochell et al., 2015). However, those studies were unable to determine whether improved growth performance was due to differences in dietary bioavailable AA content or the presence of other dietary components. Isoflavones (ISF) are flavonoid compounds that are enriched in soybeans and possess anti-inflammatory and antioxidative properties (Smith and Dilger, 2018). More specifically, under PRRSV challenge, dietary ISF have been shown to beneficially alter adaptive immune responses in weaned pigs by improving cytotoxic-to-helper T-cell ratios, which may elicit performance benefits during the recovery period (Smith et al., 2019). The objective of this experiment was to recreate immunological effects previously observed in young pigs infected with PRRSV receiving ISF and determine how those immunomodulatory effects influence growth performance during the entire growth period from weaning to market. Our hypothesis was that pigs infected with PRRSV receiving ISF would recover from PRRSV infection faster and thus exhibit better growth performance over the whole growth period.

Materials and Methods

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee and the

Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign.

Animal husbandry and experimental design

Ninety-six weanling pigs (96 barrows; 6.77 ± 0.94 kg initial BW) were obtained from a PRRS-negative, non-PRRS vaccinated commercial herd (1050 Cambro genetics; Carthage Veterinary Service, Ltd.) and group housed at the Innovative Swine Solutions Veterinary Research Facility (ISS-VRF), a Biosafety Level 2 production containment facility in Champaign, IL. Pigs remained at this facility and allowed to grow to market weight (approximately 27 wk of age, 118 to 129 kg average BW) before being transported to the Meat Science Laboratory at the University of Illinois for slaughter. The ISS-VRF consisted of 4 individual production rooms (30' × 14') with 4 pens (up to 6 pigs per pen) available to house pigs in each room. Each room was equipped with a high-efficiency particulate air filtration system, plastic slotted flooring appropriate for market-sized pigs, and each pen provided appropriate floor space for pigs taken to market weight. Lighting and ambient temperature was maintained as appropriate for life stage.

Upon entry to the facility, pigs were weighed and uniformly allotted to 1 of 3 experimental treatments [$n = 24$ for non-PRRSV-infected controls (NEG), $n = 36$ each for PRRSV-infected controls (POS) and PRRSV-infected receiving soy isoflavones (ISF)] by completely randomized design based on initial BW. All replicates of the NEG treatment (6 pigs per replicate, 4 replicates total) were housed in a single room to account for biosecurity, whereas replicates for POS and ISF treatments (6 pigs per replicate, 6 replicates total) were equally represented in the remaining 3 rooms at ISS-VRF. Two levels of supplemental ISF (none vs. $\geq 1,600$ mg/kg Novasoy400; ADM, Decatur, IL) constituted the total of 2 dietary treatments (Table 1). The ISF provided to the ISF treatment are reflective of those typical for a commercially relevant corn-soybean meal diet fed to pigs with approximately 20% soybean meal inclusion (USDA-ARS, 2016). Both diets contained soy protein concentrate as a protein source, which is practically devoid of soy isoflavones. Experimental diets were formulated to be isocaloric and, with the exceptions of corn and the isoflavone-enriched product, were identical in ingredient composition. Experimental diets were fed over 7 feeding phases including nursery phases 1 and 2 (N1 to N2) and finisher phases 1 to 5 (F1 to F5). Isoflavone and saponin concentrations of ingredients and experimental diets were quantified via HPLC at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, IL) according to procedures of Berhow et al. (2006). Crude protein was determined by measuring total nitrogen (TruMac N, Leco Corp., St. Joseph, MI) as standardized with EDTA and AA concentrations were determined at the University of Missouri Agricultural Experiment Station (Columbia, MO; Table 2) according to AOAC (2002) official methods [920.39 and 982.30 E(a, b, c), for CP and AA concentrations, respectively]. Gross energy of the experimental diets was determined using an adiabatic bomb calorimeter (Parr Instruments, Moline, IL), and DM (method

Table 1. Experimental treatments

Treatment	Dietary treatment	Infection status
NEG	Control diet	Uninfected
POS	Control diet	PRRSV infected
ISF ¹	Control diet + ISF	PRRSV infected

¹ISF provided by Novasoy 400 (ADM, Decatur, IL) at $>1,600$ mg/kg complete diet across all feeding phases.

Table 2. Ingredient and calculated composition of experimental diets (as-fed basis), nursery phases¹

Item	Nursery phase 1		Nursery phase 2	
	CON	CON + ISF	CON	CON + ISF
Ingredients, g/kg				
Corn	629.05	624.55	606.20	601.70
Soy protein concentrate ²	175.00	175.00	200.00	200.00
Dried whey	120.00	120.00	—	—
DDGS	—	—	150.00	150.00
Fish meal ³	40.00	40.00	—	—
Fat, choice white grease	10.00	10.00	10.00	10.00
Limestone	12.00	12.00	18.00	18.00
Salt	5.00	5.00	6.50	6.50
Vitamin–mineral premix ⁴	1.00	1.00	2.25	2.25
Choline chloride, 60%	0.70	0.70	0.70	0.70
Isoflavone mixture ⁵	0.00	4.50	0.00	4.50
L-Lys HCl	4.50	4.50	4.25	4.25
DL-Met	1.25	1.25	0.85	0.85
L-Thr	1.00	1.00	0.75	0.75
L-Trp	0.50	0.50	0.50	0.50
Calculated composition⁶				
ME, kcal/kg	3,470	3,455	3,449	3,434
CP, g/kg	212.4	212.0	227.6	227.2
SID AA, g/kg				
Lys	14.2	14.2	13.5	13.5
Met + Cys	7.4	7.4	11.4	11.4
Trp	2.5	2.5	2.4	2.4
Thr	8.0	8.0	7.4	7.4
Val	8.9	8.9	9.1	9.0
Isoflavones, mg/kg				
Total isoflavones	3.0	1,636	3.0	1,637
Genistein	2.0	752	2.0	753
Daidzein	1.0	789	1.0	789
Glycitein	0.0	95	0.0	95
Total saponins, mg/kg	664	1,045	759	1,140

¹All pigs received allotted treatment diet upon starting –7 DPI.

²Arcon AF, ADM, Decatur, IL.

³Special Select Menhaden Fish Meal, Omega Protein, Houston, TX.

⁴Vitamin–mineral premix (JBS United, Sheridan, IN) included the following per kilogram of complete diet: vitamin A (retinyl acetate), 11,128 IU; vitamin D3 (cholecalciferol), 2,204 IU; vitamin E (DL- α tocopheryl acetate), 66 IU; vitamin K (menadione nicotinamide bisulfite), 1.42 mg; thiamine (thiamine mononitrate), 0.24 mg; riboflavin, 6.58 mg; pyridoxine (pyridoxine hydrochloride), 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid (D-calcium pantothenate), 23.5 mg; niacin (nicotinamide and nicotinic acid), 44 mg; folic acid, 1.58 mg; biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.

⁵Novasoy400, ADM, Decatur, IL.

⁶ME and SID AA values were calculated using NRC (2012). Analyzed CP determined as CP = total N $\times$ 6.25.

934.01, AOAC International, 2002) and OM were performed by determining percent ash (method 942.05, AOAC International, 2002) and subtracting from 100. Diets were analyzed for total dietary fiber according to [Prosky et al. \(1994\)](#), but no separation of soluble and insoluble fractions was made. Diets were formulated on a standardized ileal digestible (SID) AA basis with identical concentrations across all diets ([Tables 2](#) and [3](#)). All diet formulations met or exceeded NRC (2012) nutrient recommendations for each life stage. It should be noted that after the delivery of finisher diet 3 (F3), we became aware that both diets contained less than the recommended amount of phosphorus for the first 5 feeding phases. This error impacted the Ca-to-P ratio, but this deficit was corrected in the finisher 4 (F4) and 5 (F5) diets. Analyzed nutrient composition for each diet are presented in [Tables 4](#) and [5](#).

Following a 1-wk adaptation period to experimental diets [day post-inoculation (DPI) –7 to 0], blood was collected from 2 pigs

per pen to establish 0 DPI baseline measurements and to ensure that a representative number of pigs were confirmed PRRSV-negative at experiment initiation. Immediately following blood collection, pigs were administered via intranasal inoculation either 2 mL of a 2% fetal bovine serum + PBS solution (sham-control) or 1.0×10^5 50% tissue culture infective dose of PRRS virus (strain NADC20, courtesy of Dr. Federico Zuckermann, University of Illinois, Urbana, IL).

Growth performance

Growth performance was recorded on a pen basis. Individual pig and pen feeder weights were captured weekly from –7 to 48 DPI, every other week from 48 to 118 DPI, and every third week from 118 to 161 DPI to accommodate increased heat stress associated with late summer weather during the end of the experiment for calculation of ADG, ADFI, and G:F. Growth performance data are reported in reference to our inoculation schedule (e.g., –7 to 0

Table 3. Ingredient and calculated composition of experimental diets (as-fed basis), grow-finish phases¹

Item	Finisher phase 1		Finisher phase 2		Finisher phase 3		Finisher phase 4		Finisher phase 5	
	CON	CON + ISF	CON	CON + ISF	CON	CON + ISF	CON	CON + ISF	CON	CON + ISF
Ingredients, g/kg										
Corn	535.75	531.25	664.15	659.65	815.40	810.90	826.45	821.95	884.35	879.85
Soy protein concentrate ²	125.00	125.00	100.00	100.00	100.00	100.00	90.00	90.00	85.00	85.00
DDGS	300.00	300.00	200.00	200.00	50.00	50.00	50.00	50.00	—	—
Fat, choice white grease	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Limestone	15.50	15.50	15.50	15.50	14.00	14.00	9.00	9.00	8.00	8.00
Dicalcium phosphate	—	—	—	—	—	—	8.00	8.00	7.00	7.00
Salt	5.00	5.00	4.00	4.00	4.00	4.00	2.00	2.00	2.00	2.00
Vitamin–mineral premix ³	2.50	2.50	1.50	1.50	1.00	1.00	0.75	0.75	0.75	0.75
Choline chloride, 60%	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Isoflavones ⁴	0.00	4.50	0.00	4.50	0.00	4.50	0.00	4.50	0.00	4.50
L-Lys HCl	4.50	4.50	3.25	3.25	3.25	3.25	2.30	2.30	1.50	1.50
DL-Met	0.50	0.50	0.10	0.10	0.25	0.25	—	—	—	—
L-Thr	0.25	0.25	0.50	0.50	1.00	1.00	0.50	0.50	0.50	0.50
L-Trp	0.30	0.30	0.30	0.30	0.40	0.40	0.30	0.30	0.20	0.20
Calculated composition⁴										
ME, kcal/kg	3,434	3,419	3,425	3,410	3,428	3,412	3,418	3,403	3,419	3,404
CP, g/kg	213.6	213.2	179.0	178.6	150.8	150.5	143.6	143.2	130.5	130.1
SID AA, g/kg										
Lys	11.6	11.5	9.1	9.1	8.7	8.7	7.4	7.4	6.3	6.3
Met + Cys	10.1	10.1	8.3	8.3	7.4	7.4	6.8	6.8	6.4	6.3
Trp	1.9	1.9	1.6	1.6	1.6	1.6	1.4	1.4	1.2	1.2
Thr	6.2	6.2	5.5	5.5	5.3	5.3	4.6	4.6	4.2	4.2
Val	8.2	8.2	6.9	6.9	5.9	5.9	5.6	5.6	5.2	5.1
Isoflavones, mg/kg										
Total isoflavones	2.0	1,635	2.0	1,635	2.0	1,635	2.0	1,635	1.0	1,635
Genistein	1.0	752	1.0	752	1.0	752	1.0	751	1.0	751
Daidzein	1.0	788	1.0	788	1.0	788	1.0	788	1.0	788
Glycitein	0.0	95	0.0	95	0.0	95	0.0	95	0.0	95
Total saponins, mg/kg	474	855	380	760	380	760	342	722	323	703

¹All pigs received allotted treatment diet upon starting –7 DPI.²Special Select Menhaden Fish Meal, Omega Protein, Houston, TX.³Vitamin–mineral premix (JBS United, Sheradin, IN) included the following per kilogram of complete diet: vitamin A (retinyl acetate), 11,128 IU; vitamin D3 (cholecalciferol), 2,204 IU; vitamin E (DL- α tocopheryl acetate), 66 IU; vitamin K (menadione nicotinamide bisulfite), 1.42 mg; thiamine (thiamine mononitrate), 0.24 mg; riboflavin, 6.58 mg; pyridoxine (pyridoxine hydrochloride), 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid (D-calcium pantothenate), 23.5 mg; niacin (nicotinamide and nicotinic acid), 44 mg; folic acid, 1.58 mg; biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.⁴ME and SID AA values were calculated using NRC (2012). Analyzed CP determined as CP = total N $\times$ 6.25.

DPI). Mortality was also recorded throughout the experiment, and growth performance measurements were adjusted to reflect mortalities within individual replicate pens.

Rectal temperatures

Individual pig served as the experimental unit for rectal temperatures. Rectal temperatures were recorded using a commercial-grade livestock probe thermometer (GLA M700 Thermometer, GLA Agricultural Electronics, San Luis Obispo, CA) as collected from up to 3 pigs per pen (48 pigs total, same pigs collected when possible) at 0, 3, 6, 10, and 13 DPI. If blood

collection was occurring on the same day as a rectal temperature collection, the rectal temperature was captured prior to bleeding.

Whole blood and sera collection and measurements

Individual pig served as the experimental unit for all whole blood and serum outcomes. Blood (up to 8 mL total) was collected from the jugular vein of up to 2 pigs per pen into Vacutainer blood collection tubes (BD, Franklin Lakes, NJ) at 0, 3, 6, 13, 20, 27, and 34 DPI using a 21 gauge needle. Whole blood samples collected on 0, 3, 6, and 13 DPI were collected into tubes containing EDTA as an anticoagulant, placed on ice, and submitted to the University

Table 4. Analyzed composition of experimental diets (as-fed basis), nursery phases¹

Item	Nursery phase 1		Nursery phase 2	
	CON	CON+ISF	CON	CON+ISF
DM, g/kg	909.2	911.2	903.2	900.1
OM, g/kg	932.9	936.3	946.2	937.9
CP ² , g/kg	218.3	220.3	240.3	238.2
Total dietary fiber, g/kg	99.9	117.5	156.3	156.4
Isoflavones, mg/kg				
Total Isoflavones	56.4	1,699	108	1,975
Genistein	37.9	988	87.1	1,129
Daidzein	18.5	661	22.2	785
Glycitein	0.0	49.5	0.0	61
Total saponins, ppm	193	560	442	764
Total AA, g/kg	213.0	214.6	230.4	234.1
Indispensable AA, g/kg				
Arg	12.8	12.9	9.70	14.4
His	5.40	5.50	6.20	6.30
Iso	10.0	10.1	10.6	10.7
Leu	18.5	18.7	21.3	21.7
Lys	15.1	15.5	15.3	15.5
Met	4.80	4.70	4.50	4.60
Phe	10.1	10.3	11.6	11.9
Thr	9.10	9.30	9.20	9.40
Trp	3.10	3.10	3.20	3.00
Val	11.0	10.9	11.8	12.0
Dispensable AA, g/kg				
Ala	11.1	11.2	12.3	12.5
Asp	21.0	21.0	21.8	22.3
Cys	3.30	3.30	3.90	3.80
Glu	37.2	37.3	40.8	41.5
Gly	9.70	9.70	9.80	9.90
Pro	12.5	12.5	14.6	14.8
Ser	8.50	8.60	9.30	9.70
Tyr	6.70	6.90	7.90	8.00

¹All pigs received allotted treatment diet upon starting -7 DPI.²Analyzed CP = total N × 6.25.

of Illinois College of Veterinary Medicine Clinical Pathology Lab for complete blood cell counts and differential analyses using a multiparameter, automated hematology analyzer (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL). Additional whole blood was collected on 13 and 20 DPI for a T-cell immunophenotyping protocol performed in house via flow cytometry (protocol described below). Serum samples collected on 0, 3, 6, and 13 DPI were collected into serum tubes, allowed to clot at room temperature, and centrifuged at $1,300 \times g$ for 15 min at 20 °C before storage in 0.5-mL aliquots at -80 °C pending subsequent analyses. One aliquot per pig was submitted to the Veterinary Diagnostic Laboratory for detection of PRRS viral antigen via quantitative real-time polymerase chain reaction (qRT-PCR) using Z-PRRSV Multiplex reagents (Tetracore, Rockville, MD) as described in [Smith et al. \(2019\)](#). Additional serum aliquots were separated and saved for the following assays: 1) submission to the Clinical Pathology Lab for a general health serum chemistry panel measured at 0, 3, 6, and 13 DPI and 2) submission to the University of South Dakota Animal Disease Research and Diagnostic Laboratory for detection of PRRSV-neutralizing antibodies measured at 20, 27, and 34 DPI.

T-cell immunophenotyping

Whole blood collected on 13 and 20 DPI was used for isolation of peripheral blood mononuclear cells (PBMC) for a T-cell

immunophenotyping procedure using flow cytometry. In brief, PBMC were isolated by placing individual whole blood samples over a density gradient [SepMate 15 (IVD) and Lymphoprep, StemCell Technologies, Cambridge, MA] and centrifuging at $1,200 \times g$ for 25 min at 20 °C. Isolated PBMC were then washed with phosphate buffer saline containing 2% fetal bovine serum before being counted using the Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID). Individual samples were prepared with 1.0×10^6 cells and labeled with external fluorescent antibodies against cell-surface markers CD3 (PE-Cy7 Mouse Anti-Pig CD3e, BD Pharmingen, San Jose, CA), CD4 (Alexa Fluor Mouse Anti-Pig CD4a, BD Pharmingen, San Jose, CA), and CD8 (PE Mouse Anti-Pig CD8b (BD Pharmingen, San Jose, CA). Following application of external antibodies, cells were fixed using 4% paraformaldehyde solution. Prepared, labeled cells were evaluated at the University of Illinois Flow Cytometry Facility using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, CA) and flow cytometer outputs were analyzed using FCS Express 5 Plus (De Novo Software, Glendale, CA).

Oral fluid analyses

Oral fluids were collected weekly on a pen basis starting at 39 DPI through 123 DPI or until a single pen had 2 consecutive negative test results in a row. Ropes were hung at the front of

Table 5. Analyzed composition of experimental diets (as-fed basis), grow-finish phases¹

	Finisher phase 1		Finisher phase 2		Finisher phase 3		Finisher phase 4		Finisher phase 5	
	CON ²	CON + ISF	CON	CON + ISF	CON	CON + ISF	CON	CON + ISF	CON	CON + ISF
DM, g/kg	—	888.4	881.0	879.2	864.0	840.4	867.8	867.0	874.5	866.6
OM, g/kg	—	950.1	954.2	955.3	960.8	973.1	955.0	961.0	970.5	968.5
CP ³ , g/kg	—	202.4	171.6	156.1	136.9	133.0	135.5	139.1	114.1	114.1
Total dietary fiber, g/kg	—	180.3	163.2	161.6	131.1	143.2	133.9	128.1	137.5	119.9
Isoflavones, mg/kg										
Total isoflavones	—	2,528	105	2,172	83.5	2,172	58.4	1,886	78.9	1,407
Genistein	—	1,375	84.8	1,173	66.0	1,163	48.1	1,010	64.8	753
Daidzein	—	1,064	20.2	923	17.5	928	10.3	809	14.1	605
Glycitein	—	89.4	0.0	75.0	0.0	76.7	0.0	66.7	0.0	49.9
Total saponins, ppm	—	938	345	852	406	832	250	665	265	571
Total AA, g/kg	—	187.9	172.0	164.5	141.1	132.8	142.8	132.2	123.1	119.1
Indispensable AA, g/kg										
Arg	—	10.1	9.70	9.10	8.20	7.50	8.30	7.50	7.10	6.70
His	—	5.10	4.80	4.60	3.80	3.60	3.90	3.60	3.40	3.20
Ile	—	8.20	7.50	7.00	6.20	5.70	6.30	5.80	5.40	5.20
Leu	—	19.3	17.2	16.8	14.0	13.1	13.9	13.3	12.0	11.8
Lys	—	12.3	10.3	10.0	8.60	8.60	8.90	7.80	7.20	7.10
Met	—	3.50	3.20	2.90	2.50	2.30	2.50	2.20	2.00	1.90
Phe	—	9.40	8.50	8.20	7.10	6.60	7.20	6.60	6.20	5.90
Thr	—	7.30	6.90	6.60	5.80	5.60	5.60	5.30	5.00	4.80
Trp	—	2.10	2.00	1.80	1.60	1.50	1.50	5.40	1.30	1.40
Val	—	9.60	9.00	8.50	7.30	6.60	7.30	6.70	6.20	5.90
Dispensable AA, g/kg										
Ala	—	11.3	10.4	10.1	8.20	7.80	8.20	7.80	7.10	7.00
Asp	—	16.2	14.9	14.1	12.5	11.6	13.0	11.5	11.1	10.6
Cys	—	3.30	3.20	3.00	2.50	2.30	2.60	2.40	2.20	2.10
Glu	—	32.4	29.6	28.3	25.6	23.6	25.6	23.7	22.3	21.4
Gly	—	7.90	7.50	7.10	5.90	5.50	6.00	5.60	5.10	4.90
Pro	—	13.6	12.5	12.0	9.30	9.00	9.40	8.90	8.50	8.30
Ser	—	8.20	7.00	6.90	5.70	5.70	5.90	5.40	5.10	4.90
Tyr	—	6.20	5.60	5.40	4.40	4.00	4.50	4.30	3.70	3.70

¹All pigs received allotted treatment diet upon starting –7 DPI. W/W% = grams per 100 grams of sample.²Missing sample, unable to analyze.³Analyzed CP = total N × 6.25.

each pen in 2 locations and pigs were allowed to chew on the ropes for 15 to 20 min or until the ropes were saturated with saliva. Saliva was manually expressed from the ropes into a clean plastic bag and transferred to 15-mL conical tubes for submission to the University of Illinois College of Veterinary Medicine Veterinary Diagnostic Laboratory for detection of PRRS viral antigen via qRT-PCR. Samples that could not be submitted the same day were stored temporarily at –20 °C pending analysis.

Statistical analyses

Statistical analysis was dependent on whether outcomes were measured at a single time point or at multiple time points for the same subject. For calculations of ADG, ADFI, G:F, and oral fluid samples, individual pens holding up to 6 pigs served as the experimental unit. For BW, all immune and clinical outcomes, and mortality, individual pig was considered the experimental unit, with 12 to 18 individual pigs utilized for each of the 3 experimental treatments. A total of 2 different diets were fed to PRRSV-infected pigs, while a single group of uninfected pigs received only the control diet. Thus, the 1- or 2-way analyses of variance (ANOVA) described below were performed to evaluate effects between diet treatments.

For all single time-point outcomes, a 1-way ANOVA was conducted using the MIXED procedure of SAS Enterprise Guide (version 6.1) (SAS Institute, Inc., Cary, NC). For repeated measures, a 2-way ANOVA including a time effect was conducted for all outcomes involving samples collected from the same subject at multiple time points. When appropriate, means separation was conducted. Least-square means and the SEM estimates were derived from the 1- and 2-way ANOVA. In all cases, outliers were identified as having an absolute Studentized residual value of 3 or greater and statistical significance was considered at $P \leq 0.05$.

Results

Growth performance

Results for growth performance can be found in Fig. 1 and Table 6. Between NEG and POS treatments, PRRSV infection resulted in decreased individual BW starting at 13 DPI ($P < 0.05$) through to the end of the live-phase of the experiment (161 DPI). Regarding growth performance metrics, PRRSV infection decreased ADG during growth periods 0 to 6 DPI, 6 to 13 DPI, and 13 to 20 DPI ($P < 0.05$) and decreased ADFI during growth periods 6 to 13 DPI and 13 to 20 DPI ($P < 0.05$). However, PRRSV-infected

Figure 1. Average BW of pigs by treatment over the course of the entire growth period. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Treatment ID: NEG = control diet + uninfected; POS = control diet + PRRSV infection; ISF = control diet + ISF + PRRSV infection.

pigs relative to noninfected controls only exhibited reductions in feed efficiency during the 0 to 6 DPI growth period ($P < 0.05$) and again much later during the 104 to 118 DPI growth period ($P < 0.05$).

Between POS and ISF treatments, there were differences observed for individual BW ($P < 0.0001$). Ingestion of supplemental ISF resulted in decreased BW during the later portion of the growth period (104, 118, and 161 DPI; $P < 0.001$), but no differences were observed during the acute infection or early recovery phases and the difference at 161 DPI was not maintained when pigs were weighed a few days later prior to slaughter at the University of Illinois Meat Science Laboratory. Regarding growth performance metrics, ISF supplementation increased ADFI during the 0 to 6 DPI growth period ($P < 0.05$) and the 90 to 104 DPI growth periods, but decreased ADFI during the 76 to 90 DPI ($P < 0.05$). Moreover, ISF supplementation decreased growth efficiency, as measured by G:F, during the 90 to 104 DPI growth period ($P < 0.05$), but increased growth efficiency during the 104 to 118 DPI growth period ($P < 0.05$). Across the entire growth period, however, all effects of ISF supplementation on growth performance were transient and inconsistent with the exception of individual BW.

Rectal temperatures

Results for rectal temperature can be found in [Table 7](#). Between NEG and POS treatments, PRRSV infection increased rectal temperatures significantly on 6 DPI ($P < 0.05$) with rectal temperatures of infected pigs returning to the normal range by 13 DPI, indicating successful establishment of infection. Between POS and ISF treatments, there were observed differences in rectal temperatures ($P < 0.05$), with ISF supplementation causing increased rectal temperatures at 0 DPI and decreased rectal temperatures at 6 DPI ($P < 0.05$).

Complete and differential blood cell counts

For the sake of clarity, results for the erythrogram and leukogram analyses are presented and discussed separately.

Erythrogram results

Compared with the NEG treatment, PRRSV infection resulted in decreased red blood cell (RBC) counts, decreased hemoglobin concentrations, and decreased hematocrit on 6 and 13 DPI ([Fig. 2](#)) and decreased mean cellular hemoglobin concentrations on 6 DPI ($P < 0.05$). These findings are indicative of an active infection, with many immune challenges resulting in decreased RBC synthesis and release into circulation. Between POS and ISF treatments, ISF supplementation resulted in decreased RBC counts and hematocrit at 13 DPI ($P < 0.05$). Full panel results for the erythrogram analysis can be found in [Supplementary Table 1](#).

Leukogram results

Between NEG and POS treatments, PRRSV infection resulted in decreased white blood cell (WBC) counts on 3, 6, and 13 DPI, increased proportions of circulating neutrophils at 6 and 13 DPI, and decreased proportions of circulating lymphocytes at 6 and 13 DPI ($P < 0.05$) ([Fig. 3](#)), which is in agreement with our immunophenotyping data (see below). When looking at the total counts for neutrophils, however, we observed that the POS and ISF treatments actually had fewer circulating neutrophils at 3 DPI compared with noninfected controls ($P < 0.05$), which was maintained for the POS treatment at 6 DPI. These numbers increased for our POS treatment by 13 DPI, but supplemental ISF resulted in greater numbers of circulating neutrophils to levels similar to or greater than that of our negative controls at 6 and 13 DPI. Total lymphocyte counts were consistent with the calculated proportions observed. The neutrophilia was unexpected for a viral infection model; however, due to the high incidence of secondary bacterial infections observed for this experiment, it could be considered a reasonable finding. More interestingly, the difference in total neutrophil counts across the acute infection period could be worth noting in the context of these bacterial insults following PRRSV infection. Full panel results for the leukogram analysis can be found in [Supplementary Table 2](#).

Table 6. Effects of soy isoflavones and porcine reproductive and respiratory virus (PRRSV) infection on growth performance of pigs from weaning to market weight^{1,2}

	Uninfected	PRRSV infected			Model
Growth period	NEG	POS	ISF	SEM	P-value
Active infection					
–7 to 0 DPI					
ADG, g/d	112	91.2	98.8	18.0	0.694
ADFI, g/pig/d	435	427	409	38.9	0.862
G:F, g/kg	265	230	248	52.5	0.875
0 to 6 DPI					
ADG, g/d	265 ^b	107 ^a	128 ^a	43.0	0.010
ADFI, g/pig/d	434 ^a	537 ^b	854 ^c	52.8	<0.001
G:F, g/kg	574 ^b	207 ^a	142 ^a	69.0	0.003
6 to 13 DPI					
ADG, g/d	320 ^b	85.3 ^a	43.3 ^a	62.8	0.022
ADFI, g/pig/d	568 ^b	383 ^a	503 ^a	48.8	0.036
G:F, g/kg	565	298	273	84.8	0.101
13 to 20 DPI					
ADG, g/d	416 ^b	258 ^a	238 ^a	42.9	0.019
ADFI, g/pig/d	856 ^b	440 ^a	390 ^a	88.0	0.005
G:F, g/kg	508	650	797	21.0	0.582
20 to 27 DPI					
ADG, g/d	491	419	421	58.7	0.594
ADFI, g/pig/d	761	791	691	129	0.747
G:F, g/kg	625	567	623	96.9	0.833
27 to 34 DPI					
ADG, g/d	627 ^b	404 ^a	455 ^a	61.4	0.024
ADFI, g/pig/d	1,013	863	943	151	0.683
G:F, g/kg	622	518	500	10.7	0.658
Recovery					
34 to 90 DPI					
ADG, g/d	707	692	648	28.3	0.174
ADFI, g/pig/d	1,569	1,460	1,336	81.2	0.106
G:F, g/kg	450	477	487	16.6	0.278
After viral clearance					
90 to 161 DPI					
ADG, g/d	925	959	967	37.8	0.649
ADFI, g/pig/d	2,591	2,843	2,644	167	0.216
G:F, g/kg	360	342	370	18.8	0.306

¹Values represent least-square means of 17 to 36 pigs across 4 to 6 replicates per treatment. Pigs were removed from analysis for ADG, ADFI, and G:F if their BW measurement was deemed as a statistical outlier. All pigs received allotted treatment diet starting –7 DPI.

²Growth performance was calculated on a pen basis, with each pen representing one experimental replicate containing 1 to 6 pigs per pen. In the event, a replicate pen experienced net weight loss over a growth period, they were excluded from the calculation of average feed efficiency of their treatment for that growth period.

^{abc}Means without a common superscript letter differ ($P < 0.05$).

Table 7. Effects of soy isoflavones and porcine reproductive and respiratory virus (PRRSV) infection on rectal temperature (°C) of pigs¹

Day	Uninfected	PRRSV infected		SEM	Model
	NEG	POS	ISF		P-value
Post-inoculation					
DPI 0	39.5 ^b	39.4 ^b	39.0 ^a	0.164	0.042
DPI 3	39.3	39.7	39.5	0.164	0.144
DPI 6	39.3 ^a	40.0 ^b	39.5 ^a	0.164	0.005
DPI 10	39.2	39.6	39.3	0.164	0.141
DPI 13	39.3	39.6	39.6	0.164	0.344

¹Values represent least-square means of 12 to 18 pigs. All pigs received allotted treatment diet starting –7 DPI.

^{ab}Means without a common superscript letter differ ($P < 0.05$).

Figure 2. Erythrogram results for red blood cell counts of whole blood, hemoglobin concentrations of whole blood, and percent hematocrit of whole blood collected on 0, 3, 6, and 13 d post-inoculation by treatment. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Treatment ID: NEG = control diet + uninfected; POS = control diet + PRRSV infection; ISF = control diet + ISF + PRRSV infection.

Figure 3. Leukogram results for white blood cell counts, raw neutrophil cell counts, and raw lymphocyte cell counts of whole blood collected on 0, 3, 6, and 13 d post-inoculation by treatment. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Treatment ID: NEG = control diet + uninfected; POS = control diet + PRRSV infection; ISF = control diet + ISF + PRRSV infection.

Table 8. Effects of soy isoflavones and porcine reproductive and respiratory virus (PRRSV) infection on serum viral load (Ct value) in weanling pigs¹

Day	Uninfected	PRRSV infected		Model	
Post-inoculation	NEG	POS	ISF	SEM	P-value ²
DPI 0	ND	ND	ND	0.957	1.000
DPI 3	ND	14.50	13.65	0.998	0.358
DPI 6	ND	13.98	15.50	0.998	0.621
DPI 13	ND	17.12	18.71	0.923	0.629

¹Values represent least-square means of 12 to 18 pigs. All pigs received allotted treatment diet starting -7 DPI. ND = nondetectable.

²P-values represented were calculated on log² transformed data.

Serum viral load, anti-PRRSV antibody titers, and neutralizing anti-PRRSV antibody titers

Results for serum PRRS viral antigen concentrations can be found in Table 8. Between NEG and POS treatments, PRRSV infection resulted in viremia detectable by serum PRRS viral antigen by qRT-PCR at 3, 6, and 13 DPI. Noninfected control pigs remained PRRSV-negative for serum PRRS viral antigen throughout the course of the experiment. Between POS and ISF treatments, there was no difference observed for serum PRRS viral antigen concentrations at any time point measured ($P > 0.05$).

Results for PRRSV-neutralizing antibody titers can be found in Fig. 4. Across all time points measured, the NEG treatment tested negative for PRRSV-neutralizing antibodies (i.e., titer of <1:4). At 20 DPI, 8 out of 11 POS pigs collected had negative titers, 2 had titers of 1:4, and 1 had a titer of 1:8. Within the ISF treatment, 8 out of 12 pigs collected had negative titers and 4 had titers of 1:4. At 27 DPI, 3 of the 12 POS pigs collected had negative titers, 1 had a titer of 1:4, 4 had titers of 1:8, 1 had a titer of 1:16, 2 had titers of 1:32, and 1 had a titer of 1:128. Within the ISF treatment, none of the 12 pigs collected tested negative, 5 had titers of 1:4, 4 had titers of 1:8, 1 had a titer of 1:16, and 2 had titers of 1:32. At 34 DPI, none of the 11 POS pigs collected tested negative, 1 pig had a titer of 1:4, 2 had titers of 1:8, 4 had titers of 1:16, 3 had titers of 1:32, and 1 had a titer of 1:64. Within the ISF treatment, none of the 12 pigs collected tested negative, 2 had titers of 1:8, 6 had titers of 1:16, and 4 had titers of 1:32. When looking specifically at pigs that had clinically protective titers (i.e., $\geq 1:8$), 9% (1/11) of pigs collected in the POS treatment had protective titers at 20 DPI vs. 0% (0/12) in the ISF treatment. At 27 DPI, 72% (8/11) of pigs collected in the POS treatment had protective titers vs. 58% (7/12) in the ISF treatment. At 34 DPI, 83% (10/12) in the POS treatment had protective titers vs. 100% (12/12) in the ISF treatment.

PRRSV oral fluid clearance

Results for PRRSV clearance in oral fluids by treatment can be found in Fig. 5. Monitoring of PRRSV clearance in oral fluids on a pen basis began on 39 DPI and were continued on a weekly basis through 123 DPI. The NEG treatment remained negative for oral PRRSV antigen by qRT-PCR throughout the course of the experiment. The POS treatment peaked with 100% of pens being oral-fluid-positive at 60 DPI and experienced an initial wave of clearance at 74 DPI. The ISF treatment peaked later at 67 DPI and experienced an initial wave of clearance at 81 DPI. Both POS and ISF treatments had replicate pens that reentered an infectious stage and cycled between 0% and 33% positive until

Figures 4. Counts of individual pigs within treatment with detectable anti-PRRSV neutralizing antibody titers and associated titer result at 20, 27, and 34 DPI. Antibody titers were measured in 12 pigs per treatment and the same individual pigs were used for sample collection when possible. NEG treatment was omitted from these figures as they never tested positive for anti-PRRSV antibodies across all the time points measured.

102 DPI, with a greater number of ISF replicate pens maintaining their negative status compared with the POS treatment. The ISF treatment reached final clearance at 102 DPI, whereas the POS treatment did not reach final clearance until 123 DPI.

Secondary bacterial infections and mortality prevalence

Some level of infection-related mortality can be expected among young pigs infected with PRRSV. However, starting at approximately 10 to 12 DPI, there was an observable increase in infection-related mortalities observed in our POS and ISF treatment groups, over that which was expected for this model. Based on clinical presentation and rapid onset of symptoms,

Figure 5. Visual representation of PRRSV status on pen basis by PRRS viral antigen via qRT-PCR in group-based oral fluids from 39 DPI onward. Oral fluids were collected from up to 6 pens per treatment and collection was discontinued for an individual pen following 2 consecutive weeks of negative test results. The NEG treatment was omitted from this figure as no NEG pens tested positive for PRRS viral antigen over the course of the experiment. Treatment ID: POS = control diet + PRRSV infection; ISF = control diet + ISF + PRRSV infection.

the veterinarian overseeing ISS-VRF concluded that our PRRSV-infected pigs were suffering from secondary bacterial co-infection (likely *Streptococcus suis*, though a differential diagnosis of *Haemophilus parasuis* was also suggested) (Gottschalk, 2020; Segales, 2020). Due to the large number of infected pigs impacted by this secondary infection and the increasing number of mortality cases, researchers opted to treat all pigs on study (including uninfected pigs in the NEG treatment) with a 3-d course of enrofloxacin (Baytril 100, Bayer Healthcare LLC, Shawnee Mission, KS) starting at 21 DPI in attempt to slow infection-related losses.

Results for mortality rate and extent by treatment can be found in Fig. 6. Of the original 96 pigs allotted, a total of 27 (28%) died prior to the completion of the experiment. Of those 27, 3 were categorized as non-infection/pathogen related deaths: one NEG pig that tested as a false positive for PRRSV serum antigen at 6 DPI, one NEG pig that developed severe kyphosis, and one ISF pig that developed an inguinal hernia. With those excluded, the POS treatment experienced 47% mortality while the ISF treatment experienced only 25% mortality. The initial rate of mortality appeared similar between POS and ISF treatments between 7 and 21 DPI, but the rate slowed for the ISF treatment beyond that point. Please remember that all pigs were treated with antibiotics at 21 DPI in attempt to control increased losses attributed to a severe secondary infection with *S. suis*. The POS treatment continued to experience infection-related mortalities as late as 77 DPI, while the ISF treatment experienced their last infection-related mortality at 44 DPI.

T-cell immunophenotyping results

Results for T-cell immunophenotyping can be found in Table 9. Between NEG and POS treatments, PRRSV infection caused a transient leukopenia (i.e., decrease in total lymphocytes), with

increased proportions of circulating mature T-cells (CD3+), decreased proportions of cytotoxic T-cells (CD3+/CD8+), and decreased proportions of memory T-cells (CD3+/CD4+/CD8+) ($P < 0.05$) on 13 DPI. Proportions of all impacted T-cell immunophenotype parameters in POS animals returned to values comparable to uninfected controls by 20 DPI. Interestingly, ISF increased the relative proportion of memory T-cells over the NEG treatment at 20 DPI. Regarding the helper:cytotoxic T-cell ratio (a measure of immune system functionality), PRRSV-infected treatments exhibited an increased ratio, which is typically a predictor of an appropriate immune response, with the ISF treatment having the greatest ratio value ($P < 0.05$).

Serum chemistry results

Results for serum chemistry clinical analyses can be found in Supplementary Table 3. The majority of differences seen in analyte concentrations across the acute infection period (0 to 13 DPI) appear to be due to PRRSV infection alone with differences mainly observed during the 6 and 13 DPI time points ($P < 0.05$). Regarding measures of protein status, POS and ISF treatments exhibited increased creatine concentrations, decreased creatine phosphokinase activity, and decreased total blood proteins (but increased globulin proteins at 13 DPI), which together can indicate disruptions in protein metabolism and skeletal muscle deposition, with the exception of globulin proteins which mainly indicate an active immune response. Regarding serum mineral and electrolyte concentrations, POS and ISF treatments exhibited decreased calcium, potassium, and sodium concentrations and increased chloride concentrations across the acute infection period compared with the NEG treatment. PRRSV infection also reduced blood glucose levels fairly dramatically across the acute infection period, which is expected due to the increase demand for glucose during infection and decreased feed

Figure 6. Percent mortality within treatment across the entire experiment depicted in reference to the inoculation schedule. Percent mortality is out of 24 pigs total for the NEG treatment and 36 pigs total for both POS and ISF treatments. Treatment ID: NEG = control diet + uninfected; POS = control diet + PRRSV infection; ISF = control diet + ISF + PRRSV infection.

Table 9. Effects of soy isoflavones and porcine reproductive and respiratory virus (PRRSV) infection on peripheral blood T-cell immunophenotypes of weanling pig¹

	Uninfected	PRRSV infected		SEM	Model
	NEG	POS	ISF		P-value
Cellular outcome, %					
Total lymphocytes					
DPI 13	42.3 ^b	20.9 ^a	17.2 ^a	4.25	<0.001
DPI 20	50.2	45.1	41.0	4.25	0.234
Mature T-cells ³					
DPI 13	42.0 ^a	49.7 ^b	51.5 ^b	3.08	0.034
DPI 20	51.1	45.6	51.4	3.08	0.156
Helper T-cells ⁴					
DPI 13	24.9	28.5	32.4	2.55	0.069
DPI 20	21.8	20.6	23.5	2.55	0.591
Cytotoxic T-cells ⁵					
DPI 13	32.5 ^b	23.8 ^a	23.3 ^a	3.15	0.050
DPI 20	37.0	34.7	33.1	3.15	0.625
Memory T-cells ⁶					
DPI 13	11.3 ^b	7.05 ^a	7.31 ^a	1.27	0.023
DPI 20	9.03 ^a	11.5 ^{ab}	13.2 ^b	1.27	0.046
Helper:cytotoxic T-cell ratio ⁷					
DPI 13	0.83 ^a	1.19 ^a	1.53 ^b	0.143	0.001
DPI 20	0.64	0.66	0.77	0.143	0.726

^{ab}Means without a common superscript letter differ ($P < 0.05$).

¹Values represent least-square means of 12 pigs. All pigs received allotted treatment diet starting -7 DPI.

³Percent of total lymphocytes that are positive for cell-surface marker CD3.

⁴Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4, CD8, or CD4/CD8.

⁵Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD8.

⁶Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4/CD8.

⁷Calculated ratio of percent helper T-cells to percent cytotoxic T-cells.

intake. Finally, PRRSV infection resulted in several changes observed for liver enzyme serum concentrations (i.e., decreased alkaline phosphatase concentrations, decreased glutamate dehydrogenase concentrations, increased aminotransferase concentrations) across infected treatments.

There were only a few analytes at specific time points during the acute infection period that were influenced by dietary ISF. At 6 DPI, pigs in the ISF treatment group had lower blood glucose levels than the NEG and POS treatments, but did not differ from the POS treatment at any other time point measured ($P < 0.05$). At 13 DPI, pigs in the ISF treatment group had similar sodium concentrations to those in the NEG treatment ($P < 0.05$). Additionally, at 13 DPI pigs in the ISF treatment had greater

($P < 0.05$) total CO₂ serum concentrations than the NEG treatment, with the POS treatment exhibiting intermediate concentrations.

Discussion

Isoflavones are bioactive components that are found in high concentrations in soybeans and soybean-derived feedstuffs and have been shown to have antiviral activity (Wang and Murphy, 1996). This activity has been demonstrated against PRRSV specifically in live animal studies previously (Greiner et al., 2001a,b; Rochell et al. 2015; Smith et al., 2019). The purpose of this study was to help elucidate a time-dependent biological mechanism by which ISF exert immunomodulatory effects

over the entire infection period with PRRSV, both acute and recovery phases, which was not fully captured by these previous experiments.

Induction of PRRSV infection had the greatest impact on growth performance. PRRSV-infected pigs had lower BW as early as 13 DPI and final finishing weights approximately 7% lighter than that of noninfected controls. Isoflavones only had transient impacts on growth performance with no consistent trend over the growth period. Previously, the isolated isoflavones genistein and daidzein were independently found to improve ADG during the early post-inoculation period (Greiner et al., 2001a,b), but those same effects have not been consistently observed in situations where a mixture of isoflavones to mimic naturally occurring soy ISF ratios is fed. In PRRSV-challenged pigs, those receiving higher concentrations of SBM did not experience a decrease in average daily gain, a hallmark of PRRSV infection; however, researchers could not conclude if that improvement was due to increased CP in the diet or bioactive compounds such as isoflavones (Rochell et al., 2015). However, in another PRRSV-challenge study evaluating the effects of dietary SBM on AA digestibility and losses, researchers found that a 3-time increase in dietary SBM, which would have significantly increased the ISF content in experimental diets, did not affect growth performance in infected pigs (Schweer et al., 2018). Most recently in our laboratory, PRRSV-infected weaned pigs infected with the same strain of PRRSV virus utilized for this study exhibited greater feed efficiency when receiving supplemental ISF between 6 and 14 DPI, but ISF did not affect any other growth metrics over the 2-wk infection period (Smith et al., 2019). It should be noted that the PRRSV strains used in those studies are different than what was utilized in our study. In that context, findings from the current study and previous research suggest that soy isoflavones may have some influence on growth performance under PRRSV challenge, but that influence is not consistent over the entire infection period. In addition, differences in virulence of individual PRRSV strains and the severity of clinical disease they cause (Johnson et al., 2004) may be a factor in the capacity of which isoflavones are able to influence growth performance in pathogenically stressed pigs and partially explain differences observed between PRRSV-challenge studies. Regardless, although there does not seem to be a clear explanation for why the final on-site live BW for our ISF treatment were approximately 3% lower compared with the POS treatment, this appears to be a transient effect because no differences were detected between the 2 dietary treatments at time of slaughter later the same week.

Shifting the focus from growth performance to immune response and recovery outcomes, regarding rectal temperatures as an indicator of ongoing infection, pigs in the ISF treatment had increased rectal temperatures at 0 DPI but decreased rectal temperatures at 6 DPI compared with the POS treatment. Reasoning for the increased baseline, pre-inoculation rectal temperatures of ISF supplemented pigs is unclear; however, due to fact that both the POS and ISF treatments are within the normal physiological rectal temperature range for pigs of that age (38.6 to 39.5°C), the biological relevance of that difference may be considered weak. The half-degree difference between POS and ISF treatments at 6 DPI, however, is physiologically relevant, with ISF supplementation reducing rectal temperatures of infected pigs to temperatures similar to those observed in the negative control animals.

Regarding erythrocyte measures, starting at 6 DPI we observed a decline in total RBC counts and hematocrit in both infected treatments, which has been observed previously in PRRSV-challenge models (Halbur et al., 2002). By 13 DPI, the ISF treatment

had the lowest RBC counts and hematocrit values, with the POS treatment exhibiting intermediate levels between the ISF and negative control treatment. Although within normal clinical ranges, the levels of the ISF treatment group were nearing or exceeding lower cutoff values for those measures (Friendship & Henry, 1992). It is thought that a potential cause of anemia during infection may be mediated by systemic proinflammatory cytokines, which can induce erythroid hypoplasia due to increased demand for granulocyte production from myeloid progenitor cells (Halbur et al., 2002). If true, then one explanation for why ISF consumption elicited a more severe anemia could be that pigs receiving ISF experienced a heavily neutrophilic response. Such a case has been observed with higher levels of systemic inflammation, earlier during the infection period, thus creating a stronger suppression on erythrocyte differentiation (Duncan et al., 1994). This is particularly interesting in the context of our PRRSV challenge because PRRSV is considered to be immunosuppressive, especially during the early acute infection period, and the fact that pigs on study suffered severe secondary bacterial infections.

Upon initial exposure to a viral insult, tissue-resident innate immune cells such as macrophages and dendritic cells typically produce the antiviral, proinflammatory type-I interferons, interferon (IFN)- α and IFN- β (Loving et al., 2015; Bogdan, 2000). Targeting pulmonary alveolar macrophages specifically, PRRSV dampens this type-I interferon response, with peak IFN- α titers being minimally 1,000-fold lower than that observed during other common swine respiratory viruses (e.g., swine influenza virus and porcine respiratory coronavirus). It also appears to reduce the production of other key proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) (van Reeth et al., 1999). Suppression of these signaling pathways results in poor viral clearance and a transient leukopenia and lymphopenia (Shimizu et al., 1996; Albina et al., 1998; Bogdan, 2000). We observed this in PRRSV-infected pigs, where decreased WBC and lymphocyte counts were observed starting as early as 3 DPI. However, suppression of type-I interferon signaling and recruitment of adaptive immune cells does not explain differences in neutrophil counts between our 2 infected treatments, as granulocytes are not typically associated with PRRSV infection. Some of this response may be explained by the estrogenic activity of ISF and the role of estrogen receptors on immune cells, which may have affected the susceptibility of our ISF treatment to secondary bacterial infections (Smith and Dilger, 2018; Straub, 2007; Yakimchuk et al., 2013).

Due to their structural similarity to 17 β -estradiol (E2), isoflavones have the capacity to interact with estrogen receptors throughout the body as a weak E2 analog. There are 2 main types of estrogen receptors, estrogen receptor (ER)- α , and ER- β ; of the two, soy ISF preferentially bind ER- β (Andres et al., 2009). Additionally, while more typically thought to express anti-inflammatory effects, certain cell responses mediated by ER- β are more appropriately categorized as proinflammatory. ER- β can be found throughout the body, but is notably found on peripheral leukocytes, tissue macrophages, and pulmonary epithelial tissues. One proinflammatory response by ER- β activity is increased apoptotic activity. In response to local inflammation or pathogenic insult, the expression of ER- β increases on immune cells which in turn increases pro-apoptotic pathway activation (Straub, 2007; Yakimchuk et al., 2013). It is possible that the presence of circulating ISF in pigs fed ISF may have resulted in increased apoptosis of PRRSV-infected alveolar macrophages, potentially preventing spread of PRRSV to uninfected alveolar macrophages and affording them greater functionality during a secondary bacterial insult at the level of the lung. Since it is

likely that the pigs utilized in the experiment were exposed and asymptotically harboring pathogenic bacteria prior to their arrival and inoculation with PRRSV, dietary ISF may have afforded ISF pigs increased protection against bacterial insult in the face of a PRRSV infection, allowing local immune cells to recruit an appropriate neutrophil response earlier than pigs in the POS treatment. In addition, human studies suggest that increased levels of E2 increases total numbers of neutrophils in circulation and may contribute to greater phagocytic activity in those cells; being an E2 analog, ISF may be directly contributing to the rise in neutrophil counts of our ISF treatment (Bouman et al., 2005). We provide this purported mechanism but were unable to collect lung tissue from infected pigs during acute infection to assess local cytokine levels or expression or cellular damage, so we cannot confirm whether secondary bacterial infections started in the respiratory tract. We can confirm that systemic serum cytokine concentrations did not differ between infected treatments (results not shown).

Within the broad clinical picture, the impact of ISF on immune cell numbers and mobilization may be what set the stage for recovery and greater survival by our ISF treatment. By having some influence on the kinetics of the immune response to a secondary bacterial infection, pigs receiving ISF were able to begin the recovery process from PRRSV more completely than our POS treatment. This was reflected in a few of our immune measures captured. First, our ISF treatment elicited a greater proportion of memory T-cells by 20 DPI, which are typically a group of adaptive immune cells that are relatively inactive in a PRRSV infection, but are recognized as important cells for the development of immunological memory (Lanzavecchia and Sallusto, 2005; Fu et al., 2012). Additionally, though it is in contrast to previous findings by our lab (Smith et al., 2019) and not necessarily characteristic a PRRSV infection, we found PRRSV-infected treatments exhibited an increased helper:cytotoxic T-cell ratios, with the ISF treatment having the greatest ratio value across all treatments. However, immune responses to secondary bacterial infections may have played a role in influencing this outcome. Second, although it did not seem to increase the rate of neutralizing antibody production, more pigs in the ISF treatment demonstrated protective titers by our last collection point (34 DPI) compared with the POS treatment, which was approximately 10 d following antibiotic administration. Though sampled from a small number of animals, these results suggest that ISF promote greater immune protection across an infected population, which is a key factor for PRRSV clearance and recovery. Third, and interestingly, although a majority of replicate pens in the POS treatment started to test negative by oral fluids 2 wk before the ISF treatment, half of the POS replicates continued to cycle back into positive status 5 wk beyond when a majority of ISF replicate pens tested oral fluid negative for the first time. These findings together suggest that once the ISF treatment started to enter the recovery phase from PRRSV, they did so more completely and were able to reach true clearance 7 wk earlier (with the exception of one replicate pen) than the POS treatment.

While reaching PRRSV clearance earlier in the growth period is of obvious interest to swine producers, what ISF really afforded the PRRSV-challenge pigs within this experiment was fewer infection-related losses. The ISF treatment experienced approximately 25% mortality over the entire growth period compared with the POS treatment with nearly 50% mortality. These mortality patterns suggest that under PRRSV challenge with a severe secondary bacterial infection that ISF may contribute to reduced overall mortality rates of infected pigs. As a side note, we concluded that based on our mortality patterns it appears that the incorrect

formulated calcium:phosphorus ratio in the initial finisher phase diets did not contribute strongly to overall mortality as all experimental treatments were affected and we observed an appropriate level of mortality in the uninfected, control-fed group throughout the experiment. Even in the absence of any calculated production losses, we conclude that this finding may have significant financial implications for pork productions when nearly twice as many pigs were able to make it through their entire growth period when they received ISF compared with those that did not when faced with a severe pathogenic challenge.

Unfortunately, it is still not clear exactly how ISF worked biologically within this model. Although we have suggested that it may be possible that ISF acted via estrogen-signaling pathways to affect early immune responses that later affected recovery from PRRSV complicated with secondary bacterial infections, it is important to note that ISF are a weak estrogen analog, so their activity against pathogens is likely not through those pathways exclusively (Andres et al., 2009; Smith and Dilger, 2018). In addition, we must keep in mind that this was a nutritional intervention and ISF are heavily metabolized by both host and microbial enzymes in the gastrointestinal tract. More research is warranted to elucidate whether these effects were due to direct activity by circulating isoflavones or if shifts in microbial populations could also be contributing. Finally, these findings were observed under severe pathogen challenge when an isoflavone mixture was added back to the diet at levels normally found in traditional commercial swine diets. Moving forward, there is also a need to identify whether higher isoflavone concentrations or a less severe pathogenic challenges would generate more or similar protection against infection-related losses when pigs are faced with PRRSV in a commercial setting.

Implications

Under PRRSV challenge complicated by severe secondary bacterial infections, dietary soy isoflavones reduced pathogen-associated mortality by approximately 50% over the course of the entire growth period from weaning to market. While having minimal to no impact on growth performance, isoflavones appeared to alter the kinetics of early acute immune responses, impacting the timeline for PRRSV recovery and clearance. This has significant implications for pork producers; however, more research is required to further clarify the mechanism of action for these findings and if isoflavone-enriched diets would be beneficial in modern pork production systems.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Supplemental Table 1. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on the erythrogram of weanling pigs.¹

Supplemental Table 2. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on the leukogram of weanling pigs.¹

Supplemental Table 3. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on serum chemistry parameters of weanling pigs.¹

Funding

Financial support for this experiment was provided by the United Soybean Board.

Literature Cited

- Albina, E., L. Piriou, E. Hutet, R. Cariolet, and R. L'Hospitalier. 1998. Immune responses in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet. Immunol. Immunopathol.* 61:49–66. doi:[10.1016/s0165-2427\(97\)00134-7](https://doi.org/10.1016/s0165-2427(97)00134-7)
- AOAC. 2002. *Official methods of analysis*. 17th ed. Gaithersburg, MD: AOAC Int.
- Andres, A., S. M. Donovan, and M. S. Kuhlenschmidt. 2009. Soy isoflavones and virus infections. *J. Nutr. Biochem.* 20:563–569. doi:[10.1016/j.jnutbio.2009.04.004](https://doi.org/10.1016/j.jnutbio.2009.04.004)
- Berhow, M. A., S. Bin Kong, K. E. Vermillion, and S. M. Duval. 2006. Complete quantification of group A and group B soyasaponins in soybeans. *J. Agric. Food Chem.* 54:2035–2044. doi:[10.1021/jf053072o](https://doi.org/10.1021/jf053072o)
- Bogdan, C. 2000. The function of type I interferons in antimicrobial immunity. *Curr. Opin. Immunol.* 12:419–424. doi:[10.1016/s0952-7915\(00\)00111-4](https://doi.org/10.1016/s0952-7915(00)00111-4)
- Bouman, A., M. J. Heineman, and M. M. Faas. 2005. Sex hormones and the immune response in humans. *Hum. Reprod. Update* 11:411–423. doi:[10.1093/humupd/dmi008](https://doi.org/10.1093/humupd/dmi008)
- Boyd, R. D., M. E. Johnston, and C. Zier-rush. 2010. Soybean meal level modulates the adverse effect of high immune stress on growth and feed efficiency in growing pigs. *Proc. MN Nutr. Conf.* 71: 167–174.
- Duncan, I. R., K. W. Prasse, and E. A. Mahaffery. 1994. *Duncan and Prasse's Veterinary Laboratory Medicine: Clinical Pathology*. 5th ed. Edited by K. S. Latimer. West Sussex, UK: Wiley-Blackwell.
- Friendship, R. M., and S. C. Henry. 1992. Cardiovascular system, hematology, and clinical chemistry. In: A. D. Leman, B. E. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor, editors, *Diseases of swine*. 7th ed. Iowa State University Press, Ames, IA. p. 3–11.
- Fu, Y., R. Quan, H. Zhang, J. Hou, J. Tang, and W.-H. Feng. 2012. Porcine reproductive and respiratory syndrome virus induces interleukin-15 through the NF- κ B signaling pathway. *86(14):7625–7636*. doi:[10.1128/JVI.00177-12](https://doi.org/10.1128/JVI.00177-12)
- Gottschalk, M. 2020. *Streptococcus suis* infection – Generalized conditions. Merck Vet. Man. <https://www.merckvetmanual.com/generalized-conditions/streptococcal-infections-in-pigs/streptococcus-suis-infection>.
- Greiner, L. L., T. S. Stahly, and T. J. Stabel. 2000. Quantitative relationship of systemic virus concentration on growth and immune response in pigs. *J. Anim. Sci.* 78:2690–2695. doi:[10.2527/2000.78102690x](https://doi.org/10.2527/2000.78102690x)
- Greiner, L. L., T. S. Stahly, and T. J. Stabel. 2001a. The effect of dietary soy genistein on pig growth and viral replication during a viral challenge. *J. Anim. Sci.* 79:1272–1279. doi:[10.2527/2001.7951272x](https://doi.org/10.2527/2001.7951272x)
- Greiner, L. L., T. S. Stahly, and T. J. Stabel. 2001b. The effect of dietary soy daidzein on pig growth and viral replication during a viral challenge. *J. Anim. Sci.* 79:3113–3119. doi:[10.2527/2001.79123113x](https://doi.org/10.2527/2001.79123113x)
- Halbur, R. G., F. J. Pallarés, J. A. Rathje, R. Evans, W. A. Hagemoser, P. S. Paul, and X. J. Meng. 2002. Effects of different *us* isolates of porcine reproductive and respiratory syndrome virus (PRRSV) on blood and bone marrow parameters of experimentally infected pigs. *Vet. Rec.* 151:344–348. doi:[10.1136/vr.151.12.344](https://doi.org/10.1136/vr.151.12.344)
- Holtkamp, D. J., J. B. Kliebenstein, E. J. Neumann, J. J. Zimmerman, H. F. Rotto, T. K. Yoder, C. Wang, P. E. Yeske, C. L. Mowrer, and C. A. Haley. 2013. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *J. Swine Heal. Prod.* 21:72–84. doi:[10.2460/javma.2005.227.35](https://doi.org/10.2460/javma.2005.227.35)
- Johnson, W., M. Roof, E. Vaughn, J. Christopher-Hennings, C. R. Johnson, and M. P. Murtaugh. 2004. Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. *Vet. Immunol. Immunopathol.* 102:233–247. doi:[10.1016/j.vetimm.2004.09.010](https://doi.org/10.1016/j.vetimm.2004.09.010)
- Lanzavecchia, A., and F. Sallusto. 2005. Understanding the generation and function of memory T cell subsets. *Curr. Opin. Immunol.* 17:326–332. doi:[10.1016/j.coi.2005.04.010](https://doi.org/10.1016/j.coi.2005.04.010)
- Loving, C. L., F. A. Osorio, M. P. Murtaugh, and F. A. Zuckermann. 2015. Innate and adaptive immunity against porcine reproductive and respiratory syndrome virus. *Vet. Immunol. Immunopathol.* 167:1–14. doi:[10.1016/j.vetimm.2015.07.003](https://doi.org/10.1016/j.vetimm.2015.07.003)
- Prosky, L., N. G. Asp, T. F. Schweizer, J. W. Devries, and I. Furda. 1994. Determination of insoluble and soluble dietary fiber in foods and food products – Collaborative study. *J. AOAC Int.* 75:360–367. PMID:8012222.
- Rochell, S. J., L. S. Alexander, G. C. Rocha, W. G. Van Alstine, R. D. Boyd, J. E. Pettigrew, and R. N. Dilger. 2015. Effects of dietary soybean meal concentration on growth and immune response of pigs infected with porcine reproductive and respiratory syndrome virus. *J. Anim. Sci.* 93:2987–2997. doi:[10.2527/jas.2014-8462](https://doi.org/10.2527/jas.2014-8462)
- Schweer, W. P., J. F. Patience, E. R. Burrough, B. J. Kerr, and N. K. Gabler. 2018. Impact of PRRSV infection and dietary soybean meal on ileal amino acid digestibility and endogenous amino acid losses in growing pigs. *J. Anim. Sci.* 96:1846–1859. doi:[10.1093/jas/sky093](https://doi.org/10.1093/jas/sky093)
- Segales, J. 2020. Overview of Glasser's disease – generalized conditions. Merck Vet. Manual2. <https://www.merckvetmanual.com/generalized-conditions/glasser's-disease/overview-of-glasser's-disease>.
- Shimizu, M., S. Yamada, K. Kawashima, S. Ohashi, S. Shimizu, and T. Ogawa. 1996. Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. *Vet. Immunol. Immunopathol.* 50:19–27. doi:[10.1016/0165-2427\(95\)05494-4](https://doi.org/10.1016/0165-2427(95)05494-4)
- Smith, B. N., and R. N. Dilger. 2018. Immunomodulatory potential of dietary soybean-derived isoflavones and saponins in pigs. *J. Anim. Sci.* 96:1288–1304. doi:[10.1093/jas/sky036](https://doi.org/10.1093/jas/sky036)
- Smith, B. N., A. Morris, M. L. Oelschlager, J. Connor, and R. N. Dilger. 2019. Effects of dietary soy isoflavones and soy protein source on response of weanling pigs to porcine reproductive and respiratory syndrome viral infection. *J. Anim. Sci.* 97:2989–3006. doi:[10.1093/jas/skz135](https://doi.org/10.1093/jas/skz135)
- Straub, R. H. 2007. The complex role of estrogens in inflammation. *Endocr. Rev.* 28:521–574. doi:[10.1210/er.2007-0001](https://doi.org/10.1210/er.2007-0001)
- United States Department of Agriculture – Agricultural Research Service (USDA-ARS), Nutrient Data Laboratory. 2016. USDA National Nutrient Database for Standard Reference, Release 28. Version Current September 2015, slightly revised May 2016. <https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/nutrient-data-laboratory/docs/usda-national-nutrient-database-for-standard-reference/>.
- van Reeth, K., G. Labarque, H. Nauwynck, and M. Pensaert. 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory viral infections: correlations with pathogenicity. *Res. Vet. Sci.* 67(1):47–52. doi:[10.1053/rvsc.1998.0277](https://doi.org/10.1053/rvsc.1998.0277)
- Wang, H.-J., and P. A. Murphy. 1996. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem.* 44:2377–2383. doi:[10.1021/jf950535p](https://doi.org/10.1021/jf950535p)
- Yakimchuk, K., M. Jondal, and S. Okret. 2013. Estrogen receptor α and β in the normal immune system and in lymphoid malignancies. *Mol. Cell. Endocrinol.* 375:121–129. doi:[10.1016/j.mce.2013.05.016](https://doi.org/10.1016/j.mce.2013.05.016)