Energy content of soybean meal, importation of soy products, and viral detection in inoculated feed and feed ingredients

by

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Abstract

A series of experiments were conducted to evaluate the energy content of soybean meal, understand the trends of soy products being imported to the United States, and demonstrate infectivity and detection of swine viruses in swine feed and feed ingredients. For Exp. 1, 5 treatments consisting of a control diet with solvent-extracted soybean meal (SSBM) or four diets with gradually increasing (25, 50, 75, or 100%) replacement of SSBM with extruded, expelled soybean meal (MSBM) to determine the energy content of MSBM by caloric efficiency. Diets were mixed and feed to nursery pigs for 28 days. No evidence for differences ($P > 0.05$) were observed for ADG or ADFI, but increasing MSBM in the diet improved G:F and caloric efficiency of pigs (linear, $P < 0.001$). Using caloric efficiency to estimate NE of the MSBM relative to SSBM, MSBM was estimated to have a value of 2,566 kcal/kg. In conclusion, MSBM contains approximately 123% of the energy of SSBM, which improved feed efficiency when fed to nursery pigs.

Experiment 2 evaluated soy imports into the US as a whole and from foreign animal disease positive (FAD-positive) countries to determine which products are being imported in the highest quantities and to observe potential trends in imports from FAD-positive countries. Twenty-one different Harmonized Tariff Schedule (HTS) codes from the US International Trade Commission database were queried and summarized to determine quantities (metric tonnes, MT) and breakdown of different soy product types being imported into the US from 2015 to 2020. Top exporters of soy products to the US from FAD-positive countries in 2019 and 2020 were India, Argentina, and Ukraine. The risk of FAD introduction to the US through soy imports can fluctuate based on where FAD outbreaks are occurring, shipping methods, and end usage of

products. A system to monitor these factors could help make future decisions about trade and risk of FAD introduction to US swine herds.

Experiment 3 aimed to develop a benchtop viral isolation assay for porcine reproductive and respiratory syndrome virus (PRRSV) in feed and feed ingredients. Four matrix types (soybean meal, dried distiller's grains with solubles [DDGS], complete swine diet, or cell culture media) were inoculated with (PRRSV) and incubated at three temperatures (4°C, 23°C, or 37°C) with samples being processed at 1, 24, 48, and 72 hours post-inoculation (hpi). Each sample was used for viral isolation to determine infectivity and also analyzed via quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) to measure PRRSV RNA in the sample. An interaction ($P = 0.0278$) was observed for matrix × temperature × hour for live virus detected in the model, with increasing temperature and DDGS causing PRRSV to be least infectious over time. Only matrix type influenced the quantity of viral RNA detected using qRT-PCR ($P = 0.0319$). The viral control had more viral RNA detected than DDGS with soybean meal and the swine diet being intermediate. This VI model demonstrates the ability of feed and individual feed ingredients to harbor infectious virus, and storage time and temperature influence virus inactivation.

Experiment 4 aimed to evaluate 1) the effect of benzoic acid (BA) and an essential oil blend (EO) on PPRSV, porcine epidemic diarrhea virus (PEDV), and Senecavirus A (SVA) inoculated in feed and 2) the effect of EO on PEDV inoculated into a swine vitamin premix. For the first objective, four chemical treatments four treatments consisting of 0.5% BA, 0.5% BA and 200 ppm EO, 0.3% BA and 120 ppm EO, and 0.25% BA and 100 ppm EO were used in the complete feed to test the effect on detection of PRRSV, PEDV, and SVA via qRT-PCR. For the second objective, a vitamin premix with 2.68% EO and the same premix with 2.68% limestone

were tested to determine the effects on PEDV RNA detection. The inoculated feed or premix was stored for 2, 5, and 15 d post-inoculation (dpi). In the first study, PEDV and SVA had a treatment × day interaction ($P \leq 0.008$), with an effect of time on detection of PRRSV (quadratic, $P = 0.038$). In the second experiment, the use of the EO in the premix did not demonstrate a treatment × day interaction ($P = 0.962$), an effect of treatment ($P = 0.066$), or degradation over time ($P > 0.279$). The use of a BA and/or EO mitigant in this model did not provide evidence for viral mitigation through the addition of these feed additives in either feed or premix, but viral load was reduced in the feed matrix over time.

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Dedication

This thesis is dedicated to Raymond Blomme and Ralph Keller. These two men taught by example on how to live your life through strong morals and caring for your families and communities.

Chapter 1 - Using caloric efficiency to estimate the net energy value of expelled, extruded soybean meal relative to dehulled, solvent-extracted soybean meal and its effects on growth performance of nursery pigs

ABSTRACT

This study aimed to estimate the net energy (NE) value of expelled, extruded soybean meal (MSBM) relative to dehulled, solvent-extracted soybean meal (SSBM) and determine its effects on growth performance of late nursery pigs. A total of 297 pigs (DNA 241 x 600) were weaned (BW 5.10 kg) and placed into 60 pens (2 rooms of 30 pens) with 5 pigs per pen balanced by gender and weaning weight. Pigs were fed a common diet for 21 days. Then, pens of pigs (BW 9.3 kg) were randomly assigned to one of five treatments to provide 12 replications per treatment. Treatments consisted of increasing amounts of MSBM replacing SSBM in the diet (0, 25, 50, 75, 100%). All diets were fed for 28 days and were formulated to 1.30% standardized ileal digestible lysine and met or exceeded requirements for amino acids, calcium, and phosphorus. The SSBM diet was formulated to 2,421 kcal/kg and NE was not balanced between diets. Analyzed values for CP, EE, CF, and total lysine for the SSBM were 47.28%, 0.47%, 3.80%, and 3.00% while the MSBM contained 47.41%, 6.88%, 5.32%, and 2.99% respectively. The MSBM had increased values for KOH solubility and trypsin inhibitor (83.62% and 7,026 TIU/g) compared to the SSBM (73.05% and 3,011 TIU/g) while urease activity was similar between the two (0.03 and 0.02 Δ pH, respectively). Data were analyzed using Proc GLIMMIX (SAS 9.4; Cary, NC) with pen as the experimental unit and room as the blocking factor. There

was no evidence of differences in ADG and ADFI in pigs fed diets with increasing concentrations of MSBM. Pigs fed diets with increasing concentrations of MSBM had improved (linear, $P < 0.001$) G:F and caloric efficiency on an NE basis. Using caloric efficiency to estimate NE of the MSBM relative to SSBM, MSBM was estimated to have a value of 2,566 kcal/kg. In conclusion, MSBM contains approximately 123% of the energy of SSBM, which improved feed efficiency when fed to nursery pigs.

INTRODUCTION

Oil is extracted from soybeans using either solvent or mechanical extraction. Soybean meal is a byproduct of the soy oil extraction process. Soy oil is primarily extracted with the use of a hexane mixture running through soybean flakes that had previously been cracked and dehulled (Andersen, 2011). This process is capable of removing up to 97.5% of the oil contained in soybeans, leaving as little as 0.5% of oil in the remaining meal (Andersen, 2011). Because of the efficiency of oil separation, the solvent extraction process is commonly used in soybean crush facilities. For the mechanical extraction process, the oil is extracted using an expeller press. The expeller presses the flaked soybeans through a horizontal shaft using a screw. As the flakes travel through the expeller, their compression increases because the volume of the shaft decreases. Oil is allowed to escape from the expeller while the resulting soybean meal remains. The expeller process leaves about 5 to 8% oil content in the mechanical extracted soybean meal (Boeck, 2011).

Solvent extracted soybean meal (SSBM) is commonly used in swine diets to meet protein and amino acid requirements, with pigs consuming about 24% of the soybean meal used to feed livestock (Stein et al., 2008; United Soybean Board, 2018). Mechanical extracted or expelled soybean meal (MSBM) typically has decreased concentrations of essential AA compared to

SSBM but have been demonstrated to have improved standardized ileal digestibility coefficients. In addition, MSBM contains more oil which provides increased energy content of the diet (Woodworth et al., 2001; NRC, 2012). Differing soybean sources and processing can lead to intermediate fiber, fat, and energy content between NRC (2012) reported values for MSBM and SSBM. Therefore, it is necessary to accurately quantify the energy content of MSBM for use in swine diets. The objective of this study was to determine differences in growth performance of pigs fed increasing amounts of two different sources of soybean meal (SSBM and MSBM), by using changes in caloric efficiency (CE) to estimate MSBM NE value.

MATERIALS AND METHODS

The protocol used in this study was approved by the Kansas State University Institutional Animal Care and Use Committee.

Diets and experimental design

A total of five dietary treatments consisted of increasing amounts of MSBM (0, 25, 50, 75, and 100%) used to replace SSBM on a weight/weight basis. The SSBM was sourced local to Manhattan, KS from MKC (Moundridge, KS). The MSBM was sourced from a proprietary processing plant in Nebraska and was produced using high shear dry extrusion-pressing. The soybeans were dehulled to produce SSBM, but non-dehulled soybeans were used to produce MSBM. Representative samples of SSBM and MSBM were submitted to the Agricultural Experimental Station Chemical Laboratories (University of Missouri-Columbia, Columbia, MO, USA) to be analyzed for available lysine (method 975.44, AOAC, 2012), KOH solubility (Parsons et al., 1991), trypsin inhibitor activity (method 22-40, AACC, 2006), and urease activity (method 22-90.01, AACC, 2006) (Table 1.1). Also, representative samples of corn, SSBM, and MSBM were submitted for determination of total AA content (method 982.30; AOAC, 2012).

Corn, SSBM, and MSBM were also analyzed (Agricultural Experimental Station Chemical Laboratories, University of Missouri-Columbia, Columbia, MO, USA) for dry matter (method 934.01; AOAC, 2012), crude protein (method 990.03; AOAC, 2012), crude fiber (method 978.10, AOAC, 2012), ether extract (method 920.39; AOAC, 2012), calcium (method 985.01; AOAC, 2012), and phosphorus (method 985.01; AOAC, 2012).

A total of 300 pigs (DNA 241 × 600, Columbus, NE) were weaned at 21 d of age with an average bodyweight of 5.10 kg at the Kansas State University Swine Teaching and Research Center (Manhattan, KS). Pigs were weighed and assigned to pens (1.22 × 1.52 m) with 5 pigs/pen balanced by gender and body weight. Forty of the pens contained three gilts and two barrows and twenty contained three barrows and two gilts. All pigs were fed a common diet for 21 days until they weighed an average of 9.39 kg. During this transition phase, three pigs were removed due to illness leaving 297 pigs left on test. After the 21-d common diet, pens of pigs were randomly assigned to 1 of 5 dietary treatments balanced by gender, pen weight, and location within the barn for a total of 60 treatment pens and 12 replications per treatment. Pens contained a three-hole dry self-feeder and one nipple waterer to provide *ad libitum* access to feed and water.

The five dietary treatments were fed for 28-d and consisted of increasing amounts of MSBM (0, 25, 50, 75, and 100%) used to replace SSBM on a weight/weight basis. Diets were formulated based on NRC values for AA and SID coefficients for the SSBM, MSBM, and corn. Inclusion of feed-grade AA were used to balance AA between treatments. Diets were formulated to exceed the NRC (2012) requirement estimates for AA, Ca, and P and were not balanced for NE. The NRC (2012) values for NE were used for SSBM (2,087 kcal/kg, soybean meal, dehulled, solvent extracted) and corn (2,672 kcal/kg) for calculated NE in Table 1.2. Diets were provided in mash

form. Feed disappearance and pen weights were recorded each week and were used to determine ADG, average daily feed intake (ADFI), gain to feed ratio (G:F), and CE. The actual energy value of MSBM relative to SSBM was estimated based on caloric efficiency (CE), which was obtained by multiplying ADFI by kcal of NE per kg of 0% diet (2,421 kcal/kg) and dividing by ADG for each treatment. Calculating the CE of the MSBM diet against the SSBM was selected because the MSBM was intermediate in profile between NRC (2012) reported analysis for dehulled, extruded-expelled soybean meal and extruded-expelled soybean meal. To obtain an energy estimate, the energy value of MSBM was adjusted for the slope of CE to be zero.

Chemical analysis

Representative diet samples were obtained from each treatment and stored at -20°C until analysis. Samples were analyzed for dry matter (method 934.01; AOAC, 2012), crude protein (method 990.03; AOAC, 2012), crude fiber (method 978.10; AOAC, 2012), and ether extract (method 920.39; AOAC, 2012) at Experiment Station Chemical Laboratories (Columbia, MO, USA).

Statistical Analysis

Data were analyzed as a generalized randomized complete block design with pen as the experimental unit and barn/room as the blocking factor using the PROC-GLIMMIX procedure of SAS (SAS Institute, Inc., Cary, NC). Single degree-of-freedom contrasts were constructed to test the linear and quadratic effects of increasing MSBM. Results were considered significant at $P \leq 0.05$, and marginally significant at $P \leq 0.10$.

RESULTS AND DISCUSSION

Chemical analysis

The analyzed values for corn were similar to expected values based on NRC (2012) (Table 1.1). The SSBM had decreased ether extract (0.47%) compared to the expected NRC (2012) value (1.52%). Analyzed values for the MSBM were similar to reported values for dry matter, crude fiber, Ca, and P compared to expelled soybean meal values reported in NRC (2012). However, the MSBM had increased concentrations of EE, CP, and AA compared to reported values (NRC, 2012). Although the MSBM used in this experiment was not dehulled prior to expelling, the analyzed values for EE, CP, and AA were similar to soybean meal, dehulled, expelled (NRC, 2012); however, the analyzed values for MSBM still had increased crude fiber (5.32% vs 3.30%), CP, and AA values. The analyzed crude protein and amino acids concentrations were similar between the SSBM and MSBM. The MSBM had a higher DM content than the SSBM by three percentage points. This increase in DM allowed for the MSBM to have a higher fiber value (retained hulls) but still have a similar nutrient profile to the SSBM for several nutrients. Baker and Stein (2009) observed increased ADF and NDF concentrations in extruded-expelled soybean meal as compared to solvent-extracted soybean meal as well. Trypsin inhibitor activity, KOH solubility, and urease activity were all lower for SSBM (3,011 TIU/g, 73.05%, and 0.02 Δ pH, respectively) than in MSBM (7,026 TIU/g, 83.62%, 0.03 Δ pH, respectively). Although the trypsin inhibitor activity was greater in the MSBM compared to the SSBM, the analyzed activities were similar to those observed by Chen et al. (2020). It has also been established that soybean meals with a KOH between 73 and 88% and urease levels between 0.1 and 0.3 delta pH are considered acceptable quality (U.S. Soybean Export Council, 2006). Therefore, quality measurements for both SSBM and MSBM were within acceptable ranges.

Growth performance and NE estimate

There was no evidence of difference in ADG or ADFI between the diets (Table 1.3). However, increasing MSBM concentration of the diet improved (linear, $P < 0.001$) G:F and NE CE of pigs. Experimental diets were formulated to have similar concentrations or above the recommend concentration of standard ileal digestible AA, Ca, and standardized total tract digestible P. Therefore, this improvement in G:F was likely caused by an increase in NE provided by MSBM. These difference in NE can be attributed to the increase in EE values for MSBM. As expected, this indicates that more oil was retained during the expelling process compared to the solvent extraction process (Andersen, 2011; Boeck, 2011). Woodworth et al (2001) determined that pigs fed diets containing dry extruded-expelled soybean meal or solvent-extracted soybean meal had similar growth performance when diets were formulated to be balanced for apparent ileal digestible lysine and metabolizable energy. The authors determined that dry extruded-expelled soybean meal with hulls and without hulls had 470 and 550 kcal/kg more ME than solvent-extracted without hulls (Woodworth et al., 2001). In the experiment reported herein, CE or "productive energy" was used to estimate the NE of a feed ingredient based on the known value of another (Graham et al., 2014; Gonçalves et al., 2016). The NE system was developed to determine the energy in an ingredient that is available for a pig to use for maintenance and production. The CE or "productive energy" used herein is the energy used for growth or protein deposition. Nursery pigs have potential to deposit body protein at a high rate, resulting in extra body protein gain and associated BW gain under any additional supply of balanced proteins. However, lipid tissues of nursery pigs grow at relatively low rates contributing less to the BW gain (Zhang et al., 2020). Therefore, the different EE content between MSBM and SSBM could potentially lead to risk in using the CE approach to precisely

estimate the NE value of the two ingredients in nursery pigs. While this method does not entirely capture the energy used between lipid deposition and protein deposition in nursery pigs, it has been used previously to understand the differences in energy content of two ingredients in pigs (Cemin et al., 2020). Calculating the NE content of the MSBM based on CE, estimates that MSBM (2,566 kcal/kg) reported herein is 479 kcal/kg greater than the NRC NE value of SSBM (2,087 kcal/kg). This is similar to results observed by Velayudhan et al (2015) who found that dry extruded-expelled soybean meal had an average NE of 2,544 kcal/kg. Interestingly, this value is more similar to the NRC (2012) reported value for dehulled, expelled soybean meal (2,598 kcal/kg) than the expelled soybean meal (2,344 kcal/kg). The greater caloric value for the MSBM may be due to the dry matter difference between the MSBM (93.55%) and the SSBM (89.98%), but this would only account for 55 kcal/kg when the two soybean meals are evaluated on a dry matter basis. Although the MSBM contained a greater EE than the SSBM, the MSBM also contained more crude fiber. As a result, the amount of energy that could be attributed to EE content is unable to be quantified with the current method. Additional research also determined similar NE values with extruded-expelled soybean meal (2,552 kcal/kg) providing 494 kcal/kg of additional NE as compared to solvent extracted soybean meal (2,028 kcal/kg; Rodriguez et al., 2020).

CONCLUSION

Extruded-expelled soybean meal used in the experiment conducted herein had similar concentrations of CP and essential AA and increased EE and crude fiber compared to the solvent-extracted soybean meal. Late nursery pigs fed increasing concentrations of extruded-expelled soybean meal did not demonstrate a difference in ADG or ADFI but did have improved G:F. Caloric efficiency was improved as the amount of extruded-expelled soybean meal

increased. These results determined a NE estimate of 2,566 kcal/kg for extruded-expelled soybean meal, which is equivalent to 123% of the NE of solvent-extracted soybean meal.

Table 1.1. Proximate analysis and total amino acid content of corn, solvent-extracted soybean meal (SSBM), and expelled soybean meal (MSBM), as-fed basis[a]

Item, %	Corn	SSBM	MSBM
Dry Matter	86.56	90.01	93.55
Crude Protein	8.10	47.28	47.41
Crude Fiber	1.78	3.80	5.32
Ether Extract	2.54	0.47	6.88
Calcium	0.01	0.45	0.34
Phosphorus	0.26	0.67	0.64
KOH Solubility	-	73.05	83.62
Trypsin Inhibitor, TIU/g	-	3011	7026
Urease, Δ pH	-	0.02	0.03
Amino Acids			
Alanine	0.55	2.00	1.99
Arginine	0.37	3.36	3.46
Aspartic Acid	0.53	5.28	5.41
Cysteine	0.17	0.68	0.74
Glutamic Acid	1.48	8.56	8.70
Glycine	0.32	1.97	2.01
Histidine	0.22	1.24	1.25
Isoleucine	0.30	2.31	2.33
Leucine	0.87	3.62	3.63
Lysine	0.27	2.99	3.05
Available Lysine	-	2.93	2.96
Methionine	0.16	0.65	0.64
Phenylalanine	0.38	2.43	2.49
Proline	0.65	2.34	2.40
Serine	0.35	1.91	2.12
Threonine	0.27	1.78	1.78
Tryptophan	0.06	0.67	0.69
Tyrosine	0.18	1.67	1.69
Valine	0.38	2.38	2.36

[a]A representative sample of each ingredient was obtained, homogenized, and submitted to the Agricultural Experimental Station Chemical Laboratories (University of Missouri-Columbia, Columbia, MO, USA) for proximate and amino acid analysis.

Table 1.2. Ingredient composition of experimental diets, as-fed basis

	MSBM replacement of SSBM[a], %				
	0	25	50	75	100
Ingredient, %					
Corn	63.72	63.74	63.75	63.76	63.80
Soybean meal, dehulled solvent extracted	32.50	24.37	16.25	8.13	-
Soybean meal, expelled	-	8.13	16.25	24.38	32.50
Calcium carbonate	0.95	0.95	0.95	0.95	0.95
Monocalcium phosphate, 21.5 % P	0.95	0.95	0.95	0.95	0.95
Sodium chloride	0.35	0.35	0.35	0.35	0.35
L-lysine-HCl	0.48	0.47	0.46	0.46	0.45
DL-methionine	0.24	0.23	0.21	0.19	0.17
L-threonine	0.22	0.22	0.23	0.23	0.22
L-tryptophan	0.04	0.04	0.05	0.05	0.06
L-valine	0.12	0.12	0.12	0.12	0.12
Trace mineral premix[b]	0.15	0.15	0.15	0.15	0.15
Vitamin premix[c]	0.25	0.25	0.25	0.25	0.25
Phytase[d]	0.03	0.03	0.03	0.03	0.03
Total	100	100	100	100	100
Calculated analysis, %					
SID amino acids, %					
Lys	1.35	1.35	1.35	1.35	1.35
Ile:Lys	57	56	56	56	56
Leu:Lys	116	118	120	122	124
Met:Lys	39	38	38	37	36
Met+Cys:Lys	60	60	60	60	60
Thr:Lys	65	65	65	65	65
Trp:Lys	19.5	19.5	19.5	19.5	19.5
Val:Lys	70	70	70	70	70
His:Lys	37	38	38	39	40
Net energy, kcal/kg	2421	-	-	-	-
Crude protein, %	21.6	21.4	21.2	21.0	20.8
Crude fiber, %	2.5	2.5	2.4	2.4	2.3
Ca, %	0.72	0.72	0.72	0.72	0.72
STTD P, %	0.46	0.46	0.46	0.46	0.46
Analyzed values, %					
Dry matter	88.36	88.76	89.09	89.40	89.83
Crude protein	21.95	22.00	22.23	21.78	21.48
Crude fiber	2.48	2.38	2.57	2.82	2.45
Ether extract	1.39	1.50	1.98	2.62	3.05

[a]MSBM = mechanical-extracted soybean meal; SSBM = solvent-extracted soybean meal
[b]Each kilogram contains 22g Mn, 73g Fe, 73g Zn, 11g Cu, 198mg I, and 198mg Se.
[c]Premix contains a minimum of: Vitamin A 1,653,439 IU/kg, Vitamin D3 661,376 IU/kg, Vitamin E 17,637 IU/kg, Vitamin B12 13.3 mg/kg, menadione 1,323 mg/kg, riboflavin 3,307 mg/kg, d-Pantothenic acid 11,023 mg/kg, niacin 19,841 mg/kg
[d]Ronozyme® HiPhos (GT) 2700, DSM Nutritional Products, Parsippany, NJ

Table 1.3 Effects of increasing expelled soybean meal replacing solvent-extracted soybean meal on growth performance and caloric efficiency of pigs[a]

	Treatment, %MSBM[b]						Probability, *P* <	
	0	25	50	75	100	SEM	Linear	Quadratic
BW, kg								
D 0	9.4	9.4	9.3	9.3	9.3	0.20	0.899	0.882
D 28	26.4	26.6	27.3	26.8	26.6	0.54	0.672	0.335
D 0 to 28								
ADG, g	602	604	609	622	618	0.01	0.249	0.908
ADFI, g	903	888	896	883	874	0.02	0.315	0.883
G:F, g/g	666	681	680	705	708	0.01	<0.001	0.934
CE[c], kcal/kg	3,643	3,562	3,565	3,435	3,423	46.9	<0.001	0.966

[a]A total of 297 pigs with an initial BW of 9.3 kg were used in a 28-d growth trial with 5 pigs per pen and 12 replicates per treatment

[b]MSBM = mechanical-extracted soybean meal

[c]CE = caloric efficiency obtained by multiplying ADFI by NE of 0% diet (2,421 kcal/kg) and dividing by ADG for each treatment

Chapter 2 - Assessment of soy-based imports into the US and associated foreign animal disease status

ABSTRACT

Soy-based products are known to pose a viable risk to US swine herds because of their ability to harbor and transmit virus. This publication aimed to evaluate soy imports into the US as a whole and from foreign animal disease positive (FAD-positive) countries to determine which products are being imported in the highest quantities and observe potential trends in imports from FAD-positive countries. Import data were accessed through the United States International Trade Commission website (USITC DataWeb) and summarized using R (version 4.0.2, R core team, Vienna, Austria). Twenty-one different Harmonized Tariff Schedule (HTS) codes were queried to determine quantities (metric tonnes, MT) and breakdown of different soy product types being imported into the US from 2015 to 2020. A total of 78 different countries exported soy products to the US in 2019 and 2020 with top contributors being Canada (546,467 MT and 481,497 MT, respectively), India (397,858 MT and 430,621 MT, respectively), and Argentina (122,116 MT and 79,471 MT, respectively). Soy oilcake (582,273 MT) was imported in the largest quantities, followed by organic soybeans (270,194 MT) and soy oil (134,436 MT) for 2020. Of the 78 countries, 46 had cases of FAD reported through the World Organization for Animal Health (OIE) World Animal Health Information Database (WAHIS). Top exporters of soy products to the US from FAD-positive countries in 2019 and 2020 were India (397,858 MT and 430,621 MT, respectively), Argentina (122,116 MT in 2019), and Ukraine (40,293 MT and 56,392 MT, respectively). The risk of FAD introduction to the US through soy imports can fluctuate based on where FAD outbreaks are occurring, shipping methods, and end usage of

products. A system to monitor these factors could help make future decisions about trade and risk of FAD introduction to US swine herds.

INTRODUCTION

The ability of feed and feedstuffs to serve as a vector of disease for swine can have significant impacts on the United States swine industry. Feed has been associated with the US porcine epidemic diarrhea virus (PEDV) outbreak, which caused large death loss of nursery pigs and impacted pork supplies (Scott et al., 2016). Studies have shown that African swine fever virus (ASFV) contaminated feed has the ability to cause infection in pigs as well (Dee et al., 2018; Dee et al., 2014; Niederwerder et al., 2019; Scott et al., 2016). Several viruses have been shown to survive shipping models in a variety of feed ingredients including soy products, such as soybean meal and soy oilcake (Dee et al., 2018; Stoian et al., 2020). Several viruses included in this study such as African swine fever virus (ASFV), classical swine fever virus (CSFV), Ajueszky's disease (pseudorabies), and foot and mouth disease (FMDV) are foreign animal diseases in the US and of direct interest to the swine industry. Of these viruses, only pseudorabies and CSFV have been introduced to US swine herds and eradicated (Haagmans et al., 1999; Brown & Bevins, 2018b). Vaccines exist for CSFV, FMDV, and pseudorabies, but their introduction to the US would still be detrimental to the industry (Haagmans et al, 1999; Beer et al., 2007; Knight-Jones & Rushton, 2013). Of particular concern is African swine fever virus because it has never been reported in the US and does not currently have a vaccine against it. With this knowledge, it is critical to understand what feed ingredients are being imported to the US and where they originated, so the risk level of foreign animal disease introduction can be evaluated. Of particular interest are soybean meal and soy oil because of their likelihood of being added to swine diets. Soy products have an increased ability to harbor viable virus when

compared to other feed ingredients and organic soy is of particular interest because of the claim that most soy imports into the US are organic (Dee et al., 2018; National Pork Producers Council, 2020). Some work has been done to prove an analytical approach to quantify soy imports into the US but was focused only on ASFV-positive countries and not on total imports of soy products regardless of FAD status (Patterson et al., 2020). Also, understanding the ports of entry (POE) into the US for these products is beneficial for understanding the shipping time and conditions that the majority of soy products experience. This information is useful when modeling shipping conditions or for product traceability. The objectives of this paper were 1) to evaluate annual soy imports into the US by product type and determine the portion coming from countries with foreign animal disease (FAD), 2) evaluate POE into the US and determine ports handling the largest volume of products, and 3) track soy import trends in regard to imports from FAD-positive countries.

METHODS

This work looked at import records from 2015 to 2020 with a particular focus on 2019 and 2020. Product classification, quantity, country of origin, port of entry (POE), and year were obtained through the International Trade Commission Harmonized Tariff Schedule website (DataWeb, https://dataweb.usitc.gov/). Product categories are identified by unique 10-digit Harmonized Tariff Schedule (HTS) codes (Table 1). Twenty-one HTS codes associated with soy products that have potential to be used in animal feed were used to query the database. Several products, such as lecithins or butter substitutes, were included that may end up in byproducts fed to animals. Data were exported to R (version 4.0.2, R core team, Vienna, Austria) where they were refined to total imports from each country by year and product type. Each HTS code was assigned a shortened description to improve data manipulation and reporting. Because of the low

import rate of organic soy flour and meal; soy flour and meal; and soy flour and meal, not elsewhere specified or indicated (NESOI), these three HTS product categories were combined into one group in this report (Table 2). All soy oils, regardless of refinement level, were combined into one "soy oil" category because of the low volume of imports in each subsection as well. Ports of entry provided information on the types of products coming from each country through the major ports in the US. Foreign animal disease positive countries were identified for each year and based on reported cases in any country that the World Organization for Animal Health (OIE) identified as having ASFV, CSFV, FMDV, or Aujeszky's disease (pseudorabies) cases during that year in their World Animal Health Information Database (WAHIS, https://wahis.oie.int/#/dashboards/qd-dashboard). Countries that had a case of ASFV, CSFV, FMDV, or pseudorabies in their wild or domestic herds at any point in a year were considered positive and the rest were considered negative including countries with no report submitted. Foreign animal disease status for each country by year was added to the import dataset and used to determine product types and amounts sourced from countries that experienced ASFV, CSFV, FMDV, or pseudorabies in each year.

RESULTS

Annual soy imports

In 2019, soy products were imported into the US from 65 different countries. Canada (546,467 metric tonnes, MT), India (397,858 MT), and Argentina (122,116 MT) contributed the most to soy being imported into the US in 2019 (Table 2). Overall, soy oilcake (537,470 MT) was imported in the largest quantities followed by organic soybeans (270,437 MT) and soy oil (169,721 MT). Soy flour and meal (5,878 MT) was the least commonly imported ingredient with mayonnaise (14,295 MT) and lecithins (15,838 MT) following. Eight countries (Argentina,

China, India, Mexico, Moldova, Russia, Turkey, and Ukraine) within the top 10 exporters of soy to the US and several countries outside the top 10 had reported FAD cases in 2019. The products these top FAD-positive countries exported the most to the US were soy oilcake (346,065 MT), organic soybeans (240,698 MT), and bran, midds, and residues (20,594 MT).

The top 10 countries exporting soy products to the US in 2020 were very similar to the list from 2019. Primary changes were Togo and the Netherlands overtaking Moldova and Kazakhstan (Table 3). Canada (481,497 MT), India (430,621 MT), and Argentina (79,471 MT) were still the top 3 countries soy products were sourced from in 2020. Soy oilcake (582,273 MT) was imported in the largest quantity through 2020 followed by organic soybeans (270,194 MT) and soy oils (134,436 MT). China, India, Russia, and Ukraine were all reported as FAD-positive and in the top 10 soy exporters to the US in 2020. Combined, all FAD-positive countries in 2020 primarily exported soy oilcake (387,944 MT), organic soybeans (159,041 MT), and lecithins (5,000 MT) to the US.

Ports of Entry

Soy products sourced from the top ten exporters to the US in 2019 most commonly entered into the US at 13 different ports. The port that handled the largest quantity of soy products from a country was defined as the primary port of entry (POE) for that country. The secondary and tertiary POE for a country handled the second and third largest quantities, respectively, of soy products being imported to the US from that country. The top 3 countries in 2019 that the U.S. imported soy products from were Canada, Argentina, and India. Canada primarily used Detroit, MI (204,622 MT) to send soy products to the US (Table 4). Baltimore, MD handled the next largest volume for the primary POE and was utilized by Argentina (51,507 MT) and India (132,973 MT). Both China and Turkey sent most of their soy exports to the US through San

Francisco, CA (4,357 MT and 11,665 MT, respectively). New Orleans, LA was the most common primary POE for Kazakhstan (11,062 MT), Moldova (5,986 MT), Russia (14,860 MT), and Ukraine (26,218 MT). Laredo, TX was the primary POE for products coming from Mexico (17,013 MT). For secondary POE, Buffalo, NY handled the highest volume (126,913 MT) of soy imports from top 10 countries. San Francisco, CA was the secondary POE for India, which sent 110,806 MT of soy product through this port in 2019. The most common secondary POE among the top ten soy exporters to the US was Charlotte, NC with 57,788 MT coming through this port from Argentina, Kazakhstan, Russia, or Ukraine.

Comparing the POE for the top 10 soy exporters to the US in 2019 to 2020 reveals several changes across the year. Argentina, China, and Turkey all had their secondary POE from 2019 shift to their primary port in 2020 (Charlotte, NC; Los Angeles, CA; and New Orleans, LA; respectively). Argentina also utilized Houston-Galveston, TX (12,953 MT) and San Juan, PR (12,685 MT) for a large quantity of soy exported to the US. Mexico utilized Nogales, AZ (4,261 MT) secondary to Laredo, TX (18,809 MT) in 2020, bumping San Diego, CA to third with 3,827 MT. Chicago, IL handled the highest quantity of soy products being imported from the Netherlands at 1,729 MT. Russia sent its second highest quantity of soy through Seattle, WA (814 MT) in 2020 as opposed to Charlotte, NC in 2019. San Francisco, CA handled the highest quantity of imports from Togo (6,120 MT) followed closely by Norfolk, VA (4,046 MT).

The top five POE by volume were evaluated by amount of each product type imported in 2019. Detroit, MI handled the highest volume of soy products imported to the US during this year (215,755 MT), followed by Baltimore, MD (185,578 MT); San Francisco, CA (146,279 MT); Ogdensburg, NY (144,057 MT); and Buffalo, NY (127,279 MT) (Table 5). Between these ports, Detroit brought in the largest quantity of non-organic soybeans (51,183 MT) and

Baltimore handled the largest amount of organic soybeans (60,191 MT). Most of the soy oilcake entered the US through either Baltimore (119,461 MT) or San Francisco (116,264 MT). Ogdensburg, NY handled the most soy flour and meal (1,631 MT) and soy oil (80,444 MT) of these five ports. Brans, middlings, and other residues were primarily imported to the US by Buffalo, NY (19,732 MT) and Detroit (11,985 MT). In 2020, the top five POE were Detroit, MI (190,311 MT); New Orleans, LA (183,786 MT); Baltimore, MD (162,445 MT); San Francisco, CA (151,123 MT); and Buffalo, NY (146,794 MT). Most of the non-organic soybeans imported through these five ports entered the US through Detroit (53,050 MT). New Orleans lead the top 5 POE in imports of organic soybeans with 136,709 MT. Soy oilcake was imported in large quantities through all five ports, with Baltimore and San Francisco handling the most (146,912 MT and 126,820 MT, respectively). Detroit also processed the most soy flour and meal (1,781 MT) and soy oil (79,805 MT) out of the top 5 POE in 2020. Bran, middlings, and residues were only imported through Buffalo and Detroit out of these five ports as well (20,045 MT and 8,762 MT, respectively).

Imports from countries with FAD

From 2015 to 2020, imports from reported FAD-positive countries increased from 477,806 MT to 566,318 MT (Figure 1). This increase was not consistent from year to year during this time period with large increases in imports in 2017 and 2019 followed by a decrease in 2018 and 2020. The year that had the greatest quantity of soy imports from countries with FAD cases was 2019 with 657,812 MT. India, China, and Ukraine were the top exporters of soy products to the US that had a consistently positive FAD status over these six years.

From 2015 to 2020, imports from ASFV-positive countries have varied from 44,047 to 561,583 MT (Figure 2). Ukraine was the largest contributor of imports from ASFV-positive

countries from 2015 to 2018 when it was overcome by China. China exported more soy products to the US than Ukraine from 2015 through 2018, but did not have cases of ASFV reported until 2018. Imports from China decreased in 2019 and 2020, causing Ukraine and India to be the largest contributors to US imports from ASFV-positive countries in these years, respectively. In 2020, India reported positive cases of ASFV, leading to a drastic increase in imports with ASFV risk. From 2015 to 2020, Russia and Ukraine were the only countries that were ASFV-positive every year. Russia only fluctuated between 2,083 and 7,241 MT from 2015 to 2018 and then increased to 21,997 MT in 2019 and increased even more in 2020 to 65,666 MT. Ukraine exported between 31,916 MT and 103,559 MT to the US from 2015-2020 with the peak in 2015.

Soy imports from countries with CSFV outbreaks dropped slightly from 2015 to 2016 (364,409 MT to 226,642 MT) followed by a steady increase from 2016 to 2019 with a sharp decrease in 2020 (Figure 3). Ukraine was CSFV-positive in 2015, but not in 2016, resulting in the drop in imports from CSFV-positive countries between these years. India was CSFV-positive from 2015 to 2019 and the increase of imports with CSFV risk from 2016 to 2019 matches the increased quantity of exports to the US during this time period. While India had its largest year for soy exports to the US in 2020, it did not have reported cases of CSFV in this year which caused the sharp drop in imports with CSFV risk.

Imports from FMDV-positive countries demonstrated an increase from 285,146 MT to 453,149 MT from 2015 to 2019 followed by a sharp decrease to 69,019 MT in 2020 (Figure 4). Over this period, China and Russia remained FMDV-positive each year and imports from China decreased over this time and Russia gradually increased. India was FMDV-positive from 2015 to 2019 and was the largest contributor to imports with FMD risk from 2017 to 2019. India did not

have FMD cases reported in 2020, leading to Russia becoming the largest contributor in this year.

Imports from pseudorabies-positive countries demonstrated a gradual decrease from 196,771 to 146,662 MT from 2015 to 2019, followed by a sharp decline to 203 MT in 2020 (Figure 5). China and Argentina alternated as the largest contributor to imports from pseudorabies-positive countries from 2016 to 2019. Both China and Argentina demonstrated variability in soy exports to the US from 2015 to 2020 and appear to have an inverse relationship where an increase of imports from one correlated to a decrease in imports from the other. France was the only country that reported positive cases in 2020, leading to the sharp drop in imports from pseudorabies-positive countries in this year.

DISCUSSION

The soy-based HTS codes used were selected because of the product's potential to be included in swine diets in their imported state with minimal processing or their potential to be used as a byproduct from the food industry. Several product groups, such as salad dressings, are not very likely to end up in a large volume of swine diets and therefore pose less disease risk. Products like lecithins and butter substitutes also are not likely to be used in swine diets in their current forms but their inclusion in byproducts fed to livestock gives them a greater chance of being added to swine diets. The thermal processing of feedstuffs, such as bakery byproducts may kill or inactivate pathogens in the original ingredients, but recontamination of the byproduct post-processing may allow viable virus to survive. In addition, this paper identified soy imports from FAD-positive countries. These countries were determined based on reported cases in the OIE WAHIS database and could change as more information becomes available. For example, very few countries had data reported for pseudorabies in 2020 when the database was queried for

this publication. The lack of data caused several countries to be considered “negative” for pseudorabies in 2020 even though they had not reported being free of pseudorabies.

Previous research has demonstrated the ability of soybean meal to serve as a vector for active virus, and as a result is a product of interest when evaluating FAD introduction risk (Dee et al., 2018; Stoian et al., 2020). The current evaluation found organic soybean meal to be a very small portion of the imports into the US, with 6,690 MT of organic and non-organic soybean meal being imported out of 1,234,513 MT of total soy in 2020. Less than a third of that soybean meal (2,163 MT) is sourced from countries with FAD cases which could be viewed as a low probability of disease introduction, but the severity and economic impact of disease introduction is still high. With an infectious dose of $10^{6.8}$ $TCID_{50}$ for ASFV, 300 $TCID_{50}$ for FMDV, and $10^{4.2}$ $TCID_{50}$ for CSFV, even a small amount of virus entering the country can lead to infection of US swine herds (Alexandersen et al., 2002; Cowan et al., 2015; Niederwerder et al., 2019). Smaller amounts of virus in feed can still lead to infection due to pigs eating throughout the day and accumulating virus at or above that infectious level.

Although organic SBM may not be a large contributor to US soy imports, soy oilcake is imported in large quantities. Soy oilcake is the byproduct of compressing soybeans to extract the soy oil. This oilcake can then be ground into soybean meal and included in swine diets. Soy oilcake made up 47% of soy imports in 2020, with 67% of the soy oilcake being imported from reported FAD-positive countries. The largest contributor, overall and of reported FAD-positive countries, of soy oilcake being imported into the US in 2020 was India (ASFV-positive) with 387,269 MT. The current HTS codes do not differentiate between organic and non-organic soy oilcake; therefore, it was not possible to quantify the amount of this product that is organic using the USITC DataWeb. Dee et al. (2018) has shown that soy oilcake is able to harbor viable,

infectious virus through an international shipping model. The need to grind the soy oilcake down to soybean meal before use adds another point of concern. Viral contamination of feed processing facilities has been shown to occur after the handling of contaminated feed ingredients and decontamination can be difficult (Elijah et al., 2020; Huss et al., 2017). While these studies did not address the grinding step, the production of dust during this stage of processing does provide potential for one contaminated batch of soy oilcake to infect subsequent batches by contaminating the equipment and environment in the manufacturing facility. This could allow one shipment of contaminated oilcake to inoculate multiple shipments of formerly uncontaminated oilcake or other ingredients that are processed through the same facility. Organic soybeans also were a large portion of soy imports contributing 22% of the total soy imports from 2020. Being able to understand how these soybeans are being used, whether for human consumption, livestock feed, or oil extraction will be beneficial to understanding the risk of these beans introducing disease to US swine herds. Although they are imported at about 4% of the total volume of soy products, other byproducts such as bran, middlings, and residues are important to keep in mind when considering soy imports because of the possibility of these products being used as a fiber source in swine diets.

Port of entry can also be an important factor in disease risk of feed ingredients due to the variation of time required for transport. Routes that require significant land or air transport prior to reaching a seaport for trans-Atlantic or trans-Pacific shipping will have different environmental challenges to viruses and therefore require different holding times. While many imports are entering ports on the coasts, like San Francisco or Baltimore, a fair number of imports from reported FAD-positive countries are entering through southern ports like New Orleans and Charlotte, NC. These latter two ports have not been included in previous trans-

boundary shipping models and the use of water transportation to get ingredients to their final destination may have a different effect on virus survivability (Dee et al., 2018; Dee et al., 2016; Stoian et al., 2020).

From 2015 to 2019, soy imports from reported FAD-positive countries have increased. Among the top six countries with FAD outbreaks, only China, India, and Ukraine had cases every year (Figure 1). Imports from China sharply declined in 2019, but there are factors outside of the FAD status of the country that could have contributed to this. Several tariffs were placed against China in 2018 and 2019 (United States Trade Representative, Section 301- China, List 1-4) and these actions likely contributed to the sharp decline due to the inclusion of many soy products in the tariffs included on List 3. India was the largest contributor to the increase of imports from reported FAD-positive countries from 2017 to 2020. The increase in soy imports from countries with FAD cases from 2018 to 2019 follows a similar trend to the increase in imports from India in this time.

African swine fever virus and surrogates for FMDV and pseudorabies have been demonstrated to survive transboundary shipping in soy ingredients, such as soybean meal and soy oilcake (Dee et al., 2018). Pseudorabies has been eradicated from US domestic swine herds, but is currently endemic in feral swine populations (Brown et al., 2019). This eradication was possible, in part, because of the availability of a vaccine for pseudorabies, and domestic swine herds can be vaccinated if pseudorabies is re-introduced (Haagmans et al., 1999). Classical swine fever virus has been eradicated from the US since the 1970s, but this virus still exists in portions of Central and South America, Asia, and Africa (Brown & Bevins, 2018b). Vaccines against CSFV currently exist and can be utilized if CSFV is introduced into US swine populations (Beer et al., 2007). Foot and mouth disease introduction to US herds is particularly detrimental because

of the large number of species that can be affected (Knight-Jones & Rushton, 2013). A vaccine for FMDV exists, but vaccination plans will likely involve movement restrictions within a certain area surrounding an outbreak (Knight-Jones & Rushton, 2013). African swine fever is endemic in portions of Africa and Europe and currently has no vaccine (Brown & Bevins, 2018a). Introduction of ASFV to US swine herds would have a large, detrimental impact because of the lack of vaccination possibility and likelihood of trade restrictions.

Imports from ASFV-positive countries decreased from 2015 until a spike in 2018. The addition of China to the list of positive countries in 2018 was most likely the reason for this increase and positive ASFV cases in India led to an even more drastic increase in 2020. Soy products sourced from countries with CSFV outbreaks saw a decrease from 2015 to 2016 and a steady rise from 2016 to 2019 with a sharp decrease in 2020. Once again, India contributed to the rise in imports from CSFV-positive countries across this span, and the lack of reported CSFV cases in the country in 2020 led to the sharp decline of soy products imported from CSFV-risk countries. Soy imports sourced from countries with FMDV-positive status also experienced an increase from 2015 to 2019 followed by a decline in 2020. A large portion of that can be correlated to imports from India from 2016 to 2019, as that was the country that contributed the most to soy imports in this category. The decrease in imports from FMDV-positive countries in 2020 is due to India no longer having reported FMDV cases, leaving Russia to be the largest contributor of imports with FMDV-risk for this year. Pseudorabies-positive countries are the only FAD category in this investigation that decreased exports to the US from 2015 to 2019. For the first three diseases, India was a large driver in import trends. In contrast, China and Argentina contributed the most to imports with pseudorabies-risk. Argentina was one of the largest exporters of soy products to the US in 2019 and 2020, so its inclusion in pseudorabies-

positive countries had a large impact. Prior to 2017, China exported more soy products to the US than Argentina, increasing the amount of imports from countries experiencing pseudorabies outbreaks. In 2017, Argentina overtook China for the top pseudorabies-positive country and these two alternated the top position each year through 2019. In 2020, France was the only country with pseudorabies reported, so quantities of soy sourced from pseudorabies-positive countries dropped drastically. The OIE WAHIS database is updated with each report put out by the World Organization for Animal Health, whether that is a quarterly, 6-month, or annual report. Due to this fact, the FAD status of countries within this timeframe may change as countries that had no data for a year report positive cases or a negative status. As a result, numbers for 2020 will likely change with the release of more information.

Overall, imports from reported FAD-positive countries contributed about 53% of the total soy imports in 2019 with India, Argentina, and Turkey being the largest individual contributors within this group. In 2020, approximately 46% of the total soy import was sourced from reported FAD-positive countries. This high percentage is primarily due to reports of FAD in India and Argentina. Both of these were among the top three exporters of soy products to the US for each year. The trend of imports from ASFV-positive countries is a prime example of the impact of even one major soy exporter experiencing a disease outbreak. India breaking with ASFV in 2020 lead to a drastic increase in imports sourced from ASFV-positive countries. It also should be noted that this information does not take into account that the products imported from FAD-negative countries may have been imported from somewhere else previously. Similar to a statement expressed by Patterson (2020), the interconnectedness of the global economy makes it difficult to trace the original source of products in some cases. A deeper look into where a region's products are being sourced from would be beneficial in understanding the disease risk of

the product more objectively. The end use is also an important consideration because a product that is used exclusively for human or industrial consumption also has a low disease risk, even if it is contaminated, because of its removal from interaction with swine herds.

CONCLUSION

Understanding the sources and intended uses of products being imported to the US is vital to determining the risk of FAD disease introduction. Quantifying the amount and country of origin for imports into the US is beneficial to start digging deeper into the biosecurity of feed across the country. While this quantification is beneficial, it should not be taken as a defining declaration of the risk of FAD introduction without a holistic view of the storage, transport, and usage of imported soy products. Being able to monitor FAD disease outbreaks and imports from countries could be useful for evaluating the risk of FAD introduction into US swine herds more readily.

Table 2.1 HTS codes utilized, their product descriptions, and shortened names

HTS Code	Product Description	Short Name
1201.90.0005	SOYBEAN SEEDS OF A KIND USED AS OIL STOCK, WHETHER OR NOT BROKEN	Soybean Seeds
1201.90.0010	SOYBEANS, CERTIFIED ORGANIC, WHETHER OR NOT BROKEN, EXCEPT SEEDS OF A KIND USED FOR SOWING OR USED AS OIL STOCK	Organic Soybeans
1201.90.0090	SOYBEANS, WHETHER OR NOT BROKEN, OTHER THAN CERTIFIED ORGANIC, NESOI	Non-organic Soybeans
1208.10.0000	FLOURS AND MEALS OF SOYBEANS	Soy Flour and Meal
1208.10.0010	FLOURS AND MEALS OF SOYBEANS, CERTIFIED ORGANIC	Organic Soy Flour and Meal
1208.10.0090	FLOURS AND MEALS OF SOYBEANS, NESOI	Soy Flour and Meal, NESOI
1507.10.0000	SOYBEAN OIL AND ITS FRACTIONS, CRUDE, WHETHER OR NOT DEGUMMED	Crude Oil
1507.90.4020	SOYBEAN OIL AND ITS FRACTIONS, ONCE-REFINED (SUBJECT TO ALKALAI OR CAUSTIC WASH BUT NOT BLEACHED OR DEODORIZED), NOT CHEMICALLY MODIFIED	Once-refined Oil
1507.90.4040	SOYBEAN OIL AND ITS FRACTIONS, FULLY REFINED, WASHED, BLEACHED OR DEODORIZED BUT NOT CHEMICALLY MODIFIED, NESOI	Fully Refined Oil
1517.10.0000	MARGARINE, EXCLUDING LIQUID MARGARINE	Margarine
1517.90.9025	SOYBEAN OIL, WHOLLY HYDROGENATED, NESOI	Wholly Hydrogenated Oil
2103.90.9020	MAYONNAISE	Mayonnaise
2103.90.9040	SALAD DRESSINGS, NESOI	Salad Dressings
2106.90.2400	BUTTER SUBSTITUTES CONTAINING OVER 10% BY WEIGHT OF MILK SOLIDS, CONTAINING OVER 45% BUTTERFAT, SEE ADDITIONAL U. S. NOTE 14 - CHAP. 4 & PROVISIONAL	Butter Substitutes
2106.90.2600	BUTTER SUBSTITUTES CONTAINING OVER 10% BY WEIGHT OF MILK SOLIDS, CONTAINING OVER 45% BUTTERFAT, NESOI	Butter Substitutes
2106.90.2800	BUTTER SUBSTITUTES, IN LIQUID OR SOLID STATE, CONTAIN GT 15% BY WEIGHT OF BUTTER OR OTHER FATS OR OILS DERIVED FROM MILK, GT 10% MILK SOLIDS, NESOI	Butter Substitutes
2106.90.3600	BUTTER SUBSTITUTES WHETHER IN LIQUID OR SOLID STATE, NESOI, CONTAINING OVER 45 PERCENT BUTTERFAT, NESOI	Butter Substitutes
2106.90.3800	BUTTER SUBSTITUTES, WHETHER IN LIQUID OR SOLID STATE, CONTAINING OVER 15% BY WEIGHT OF BUTTER OR OTHER FATS OR OILS DERIVED FROM MILK, NESOI	Butter Substitutes
2302.50.0000	BRAN, SHARPS (MIDDLINGS) AND OTHER RESIDUES, WHETHER OR NOT IN PELLETS, DERIVED FROM SIFTING, MILLING OR OTHER WORKINGS OF LEGUMINOUS PLANTS	Brans, Midds, Residues
2304.00.0000	SOYBEAN OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF SOYBEAN OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS	Soy Oilcake
2923.20.2000	LECITHINS AND OTHER PHOSPHOAMINOLIPIDS, NESOI	Lecithins

Table 2.2 Top 10 exporters in 2019 of soy to US with products, quantities (MT), and FAD status[a,b]

		Products											
Country	Total	Non-Organic Soybeans	Organic Soybeans	Soy Oilcake	Soy Flours and Meals	Soy Oil	Bran, Midds, Residues	Lecithins	Mayonnaise	Salad Dressings	Butter and Margarine	Soybean Seeds	FAD Present in Country
Canada	546,467	75,846	13,991	190,999	2,809	163,888	41,152	4,697	10,722	3,222	7,978	31,162	No
India	397,858	8,675	80,681	304,772	1,849	615	0.0	1,265	0.0	0.8	0.5	0.0	Yes
Argentina	122,116	0.0	88,744	14,815	0.0	1,150	16,652	755	0.0	0.0	0.0	0.0	Yes
Ukraine	40,293	0.0	40,143	0.0	0.0	0.0	0.0	68	82	0.0	0.0	0.0	Yes
Turkey	23,348	0.0	456	21,973	0.0	0.0	0.0	0.0	0.0	902	17	0.0	Yes
Russia	21,997	0.0	20,661	0.0	0.0	0.0	0.0	1,064	266	0.0	6.2	0.0	Yes
Mexico	20,833	3.2	2,180	0.0	256	3,498	0.0	0.0	686	11,258	2,952	0.0	Yes
Kazakhstan	13,337	0.0	13,337	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	No
China	9,038	1,481	137	4,449	79	6.9	2,681	137	0.0	18	19	30	Yes
Moldova	5,986	0.0	5,986	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	Yes
Others[c]	32,816	345	4,120	463	885	564	2,736	7,852	2,538	2,316	10,997	0.0	Yes
Grand Total	1,234,089	86,352	270,437	537,470	5,878	169,721	63,221	15,838	14,294	17,717	21,969	31,192	NA

[a] Countries, products, and quantities (MT) were obtained from the United States International Trade and Tariff Database.

[b] Foreign animal disease status was determined based on presence of African swine fever virus, classical swine fever virus, foot and mouth disease, and/or pseudorabies virus in a country during 2019 as reported by the OIE WAHIS Disease Time Chart database.

[c] Countries included in others: Afghanistan, Australia, Austria, Bangladesh, Barbados, Belarus, Belgium, Brazil, Chile, Colombia, Costa Rica, Croatia, Denmark, Dominican Republic, Ecuador, El Salvador, Ethiopia, France, Germany, Ghana, Greece, Guatemala, Guyana, Haiti, Hungary, Indonesia, Ireland, Israel, Italy, Jamaica, Japan, Jordan, Lithuania, Malaysia, Morocco, Netherlands, Nigeria, North Macedonia, Norway, Peru, Philippines, Poland, Portugal, Serbia, Slovenia, South Korea, Spain, Sweden, Switzerland, Syria, Taiwan, Thailand, Togo, Trinidad and Tobago, United Kingdom, Uruguay, and Vietnam.

Table 2.3 Top 10 exporters in 2020 of soy to US with products, quantities (MT), and FAD status[a,b]

		Products											
Country	Total	Non-Organic Soybeans	Organic Soybeans	Soy Oilcake	Soy Flours and Meals	Soy Oil	Bran, Midds, Residues	Lecithins	Mayonnaise	Salad Dressings	Butter and Margarine	Soybean Seeds	FAD Present in Country
Canada	481,497	69,987	19,064	152,128	3,619	123,502	42,913	3,835	11,988	1,654	7,412	45,393	No
India	430,621	4.1	38,108	387,269	1,748	2,155	0.0	1,337	0.0	0.0	0.8	0.0	Yes
Argentina	79,471	160	65,898	7,546	0.0	227	5,139	501	0.0	0.0	0.8	0.0	No
Russia	65,666	0.0	64,478	0.0	0.0	0.0	0.0	818	364	0.0	7.2	0.0	Yes
Ukraine	56,392	0.0	56,093	0.0	0.0	0.0	0.0	186	113	0.0	0.0	0.0	Yes
Turkey	40,518	0.0	0.0	34,276	0.0	3,906	24	0.0	788	1,513	12	0.0	No
Mexico	27,965	18	7,518	0.0	405	2,952	0.0	0.0	814	13,661	2,598	0.0	No
Togo	11,394	318	11,076	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	No
China	3,348	1,208	153	468	33	0.0	1,269	186	10	22	0.0	0.0	Yes
Netherlands	3,222	0.0	0.0	0.0	0.0	18	12	3,162	30	0.3	0.2	0.0	No
Others[c]	34,419	1,669	7,807	588	885	1677	1,418	4,427	3,205	2,326	10,418	0.0	Yes
Grand Total	1,234,513	73,363	270,194	582,273	6,690	134,436	50,775	14,451	17,312	19,176	20,449	45,393	NA

[a] Countries, products, and quantities (MT) were obtained from the United States International Trade and Tariff Database

[b] Foreign animal disease status was determined based on prevalence of African swine fever, classical swine fever, foot and mouth disease, and/or pseudorabies in a country during 2020 as reported by the OIE WAHIS Disease Time Chart database

[c] Countries included in others: Afghanistan, Australia, Austria, Belgium, Benin, Brazil, Chile, Colombia, Costa Rica, Croatia, Czech Republic, Denmark, Dominican Republic, Ecuador, Egypt, Ethiopia, France, Germany, Greece, Guatemala, Guyana, Haiti, Hungary, Indonesia, Iran, Ireland, Israel, Italy, Jamaica, Japan, Jordan, Kenya, Lithuania, Malaysia, Morocco, Nigeria, North Macedonia, Norway, Paraguay, Peru, Philippines, Poland, Portugal, Rwanda, Senegal, Serbia, Singapore, Slovenia, South Korea, Spain, Sweden, Switzerland, Syria, Taiwan, Thailand, Trinidad and Tobago, United Arab Emirates, United Kingdom, Uruguay, Venezuela, and Vietnam

Table 2.4 Top 10 exporters of soy and primary US ports of entry with quantity (MT) in 2019 and 2020[a]

Country	Primary Port		Second Port		Third Port	
2019						
Argentina	Baltimore, MD	51,507	Charlotte, NC	36,588	San Francisco, CA	18,544
Canada	Detroit, MI	204,622	Buffalo, NY	126,913	Ogdensburg, NY	122,478
China	San Francisco, CA	4,357	Los Angeles, CA	2,464	Seattle, WA	880
India	Baltimore, MD	132,973	San Francisco, CA	110,806	Seattle, WA	67,781
Kazakhstan	New Orleans, LA	11,062	Charlotte, NC	2,275	NA	NA
Mexico	Laredo, TX	17,013	San Diego, CA	2,454	El Paso, TX	971
Moldova	New Orleans, LA	5,986	NA	NA	NA	NA
Russia	New Orleans, LA	14,860	Charlotte, NC	5,000	Ogdensburg, NY	801
Turkey	San Francisco, CA	11,665	New Orleans, LA	10,000	New York, NY	879
Ukraine	New Orleans, LA	26,218	Charlotte, NC	13,925	New York, NY	88
2020						
Argentina	Charlotte, NC	35,541	Houston-Galveston, TX	12,953	San Juan, PR	12,685
Canada	Detroit, MI	169,946	Buffalo, NY	146,466	Ogdensburg, NY	76,879
China	Los Angeles, CA	1,665	New York, NY	561	Seattle, WA	475
India	Baltimore, MD	147,110	San Francisco, CA	126,782	Seattle, WA	52,256
Mexico	Laredo, TX	18,809	Nogales, AZ	4,261	San Diego, CA	3,827
Netherlands	Chicago, IL	1,729	New York, NY	454	Los Angeles, CA	387
Russia	New Orleans, LA	63,462	Seattle, WA	814	New York, NY	613
Togo	San Francisco, CA	6,120	Norfolk, VA	4,046	Philadelphia, PA	678
Turkey	New Orleans, LA	24,214	San Francisco, CA	13,422	New York, NY	1,414
Ukraine	New Orleans, LA	51,093	Charlotte, NC	5,000	New York, NY	164

NA = not applicable

[a] Countries of origin, ports of entry, and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.

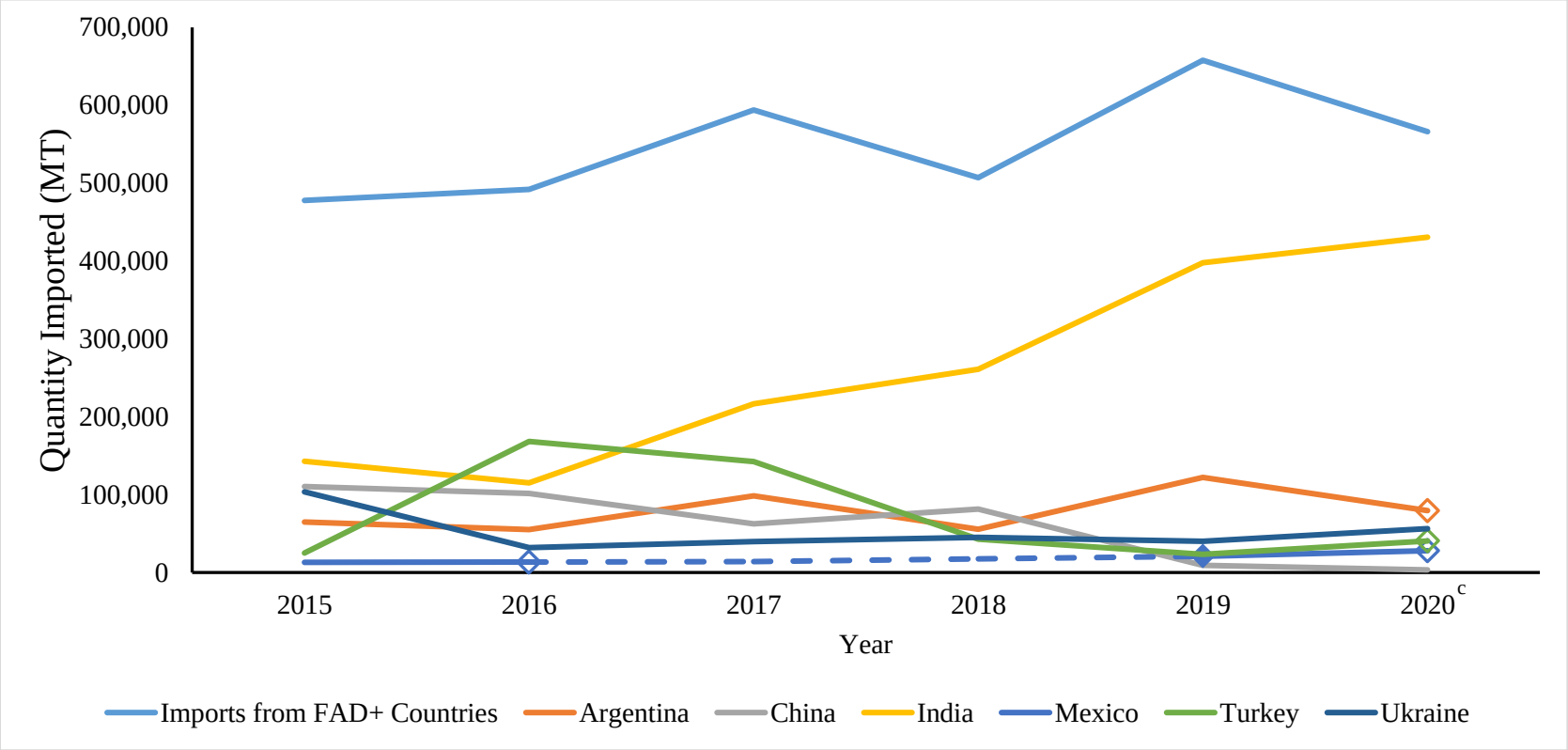
Table 2.5 Top 5 ports of entry and quantity (MT) of associated products in 2019 and 2020[a]

	Ports of Entry for 2019				
Product	Detroit, MI	Baltimore, MD	San Francisco, CA	Ogdensburg, NY	Buffalo, NY
Non-Organic Soybeans	51,183	5,679	882	5,310	5,310
Organic Soybeans	594	60,191	17,345	19,489	19,489
Soy Oilcake	68,395	119,461	116,264	35,899	71,799
Soy Flour and Meals	282	0.6	223	1,631	1.1
Soy Oils	66,115	181	1,045	80,444	5,754
Bran, Midds, Residues	11,985	0.0	9,570	149	19,732
Lecithins	4,713	15	934	18	6.9
Mayonnaise	4,990	9.5	4.4	18	5,728
Salad Dressings	1,928	41	6.8	49	1,110
Margarine	5,572	0.0	4.9	1,050	630
Soybean Seeds	0.0	0.0	0.0	0.0	43
Total	215,755	185,578	146,279	144,057	127,279

	Ports of Entry for 2020				
Product	Detroit, MI	New Orleans, LA	Baltimore, MD	San Francisco, CA	Buffalo, NY
Non-Organic Soybeans	53,050	0.0	733	1.8	9,348
Organic Soybeans	2,600	136,709	13,552	18,702	2,637
Soy Oilcake	26,706	46,904	146,912	126,820	104,278
Soy Flour and Meals	1,781	0.0	0.0	1.7	120
Soy Oils	79,805	0.0	228	4,966	4,503
Bran, Midds, Residues	8,762	0.0	0.0	0.0	20,045
Lecithins	3,894	172	67	595	43
Mayonnaise	7,982	0.0	785	22	4,002
Salad Dressings	612	0.0	169	8.6	718
Margarine	4,129	0.0	0.0	5.3	752
Soybean Seeds	989	0.0	0.0	0.0	348
Total	190,311	183,786	162,445	151,123	146,794

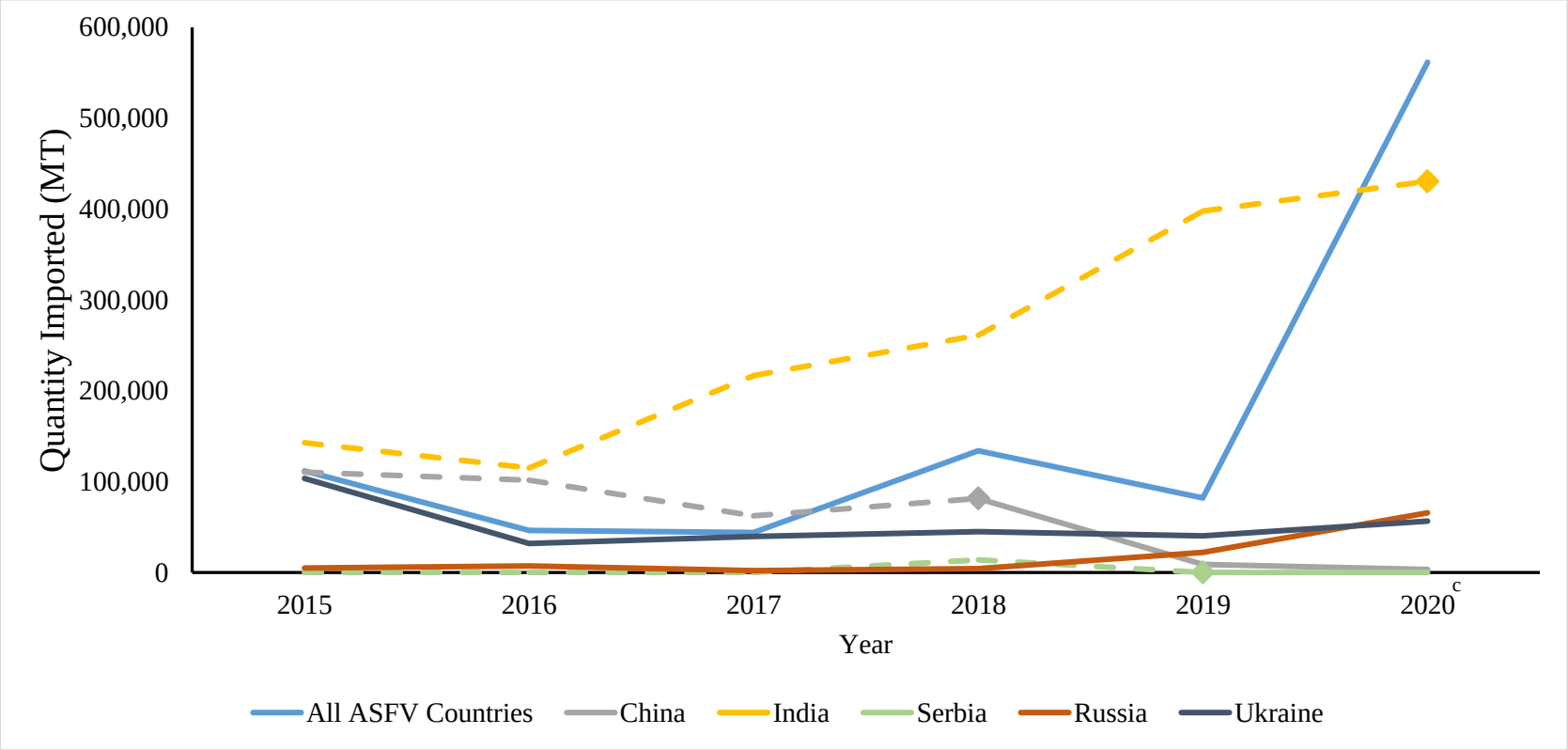
[a]Product types, ports of entry, and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database

Figure 2.1. Imports from countries with foreign animal disease from 2015 to 2020 and the top 6 exporters of soy to the US by quantity (MT)[a,b]



Dashed lines indicate years that a country did not have FAD status reported or there were no positive cases.
Diamond markers are the single year that a country had reported FAD cases.
Open diamond markers indicate the beginning of years that the country is FAD-negative or did not have reported cases.
[a] Countries of origin and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.
[b] Foreign animal disease status was determined based on presence of African swine fever virus, classical swine fever virus, foot and mouth disease, and/or pseudorabies in a country during each year as reported by the OIE WAHIS Quantitative Data database.
[c] No differentiation provided in figure between countries reporting no FAD cases and countries with no FAD data for 2020 in the OIE WAHIS Quantitative Database

Figure 2.2 Imports from countries with African swine fever virus from 2015 to 2020 and the top 5 exporters of soy to the US by quantity (MT)[a,b]



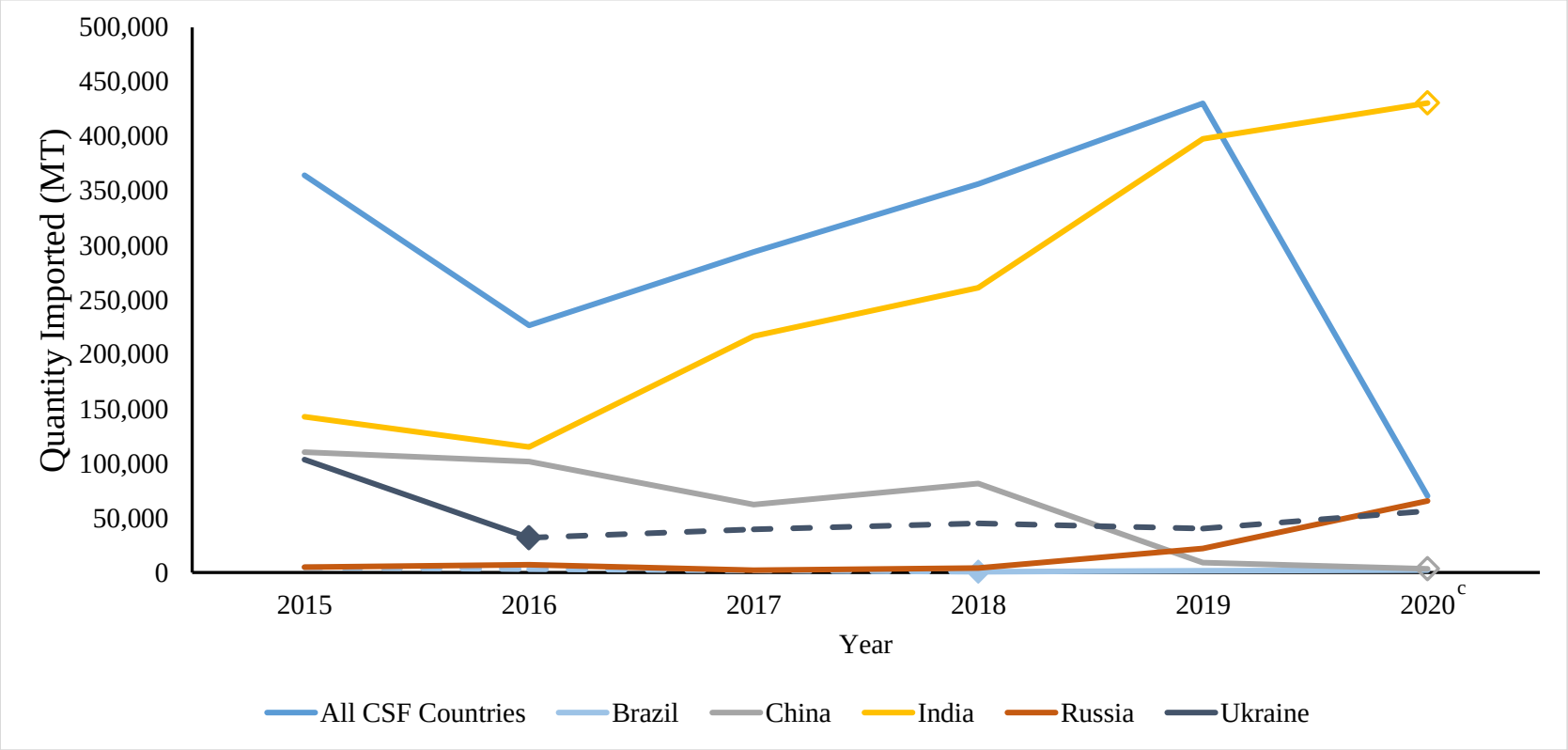
Dashed lines indicate years that a country did not have ASFV status reported or there were no positive cases.
Diamond markers are the year that a country began to have reported ASFV cases.

[a] Countries of origin and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.

[b] African swine fever status was determined based on presence of cases in a country during each year as reported by the OIE WAHIS Quantitative Data database.

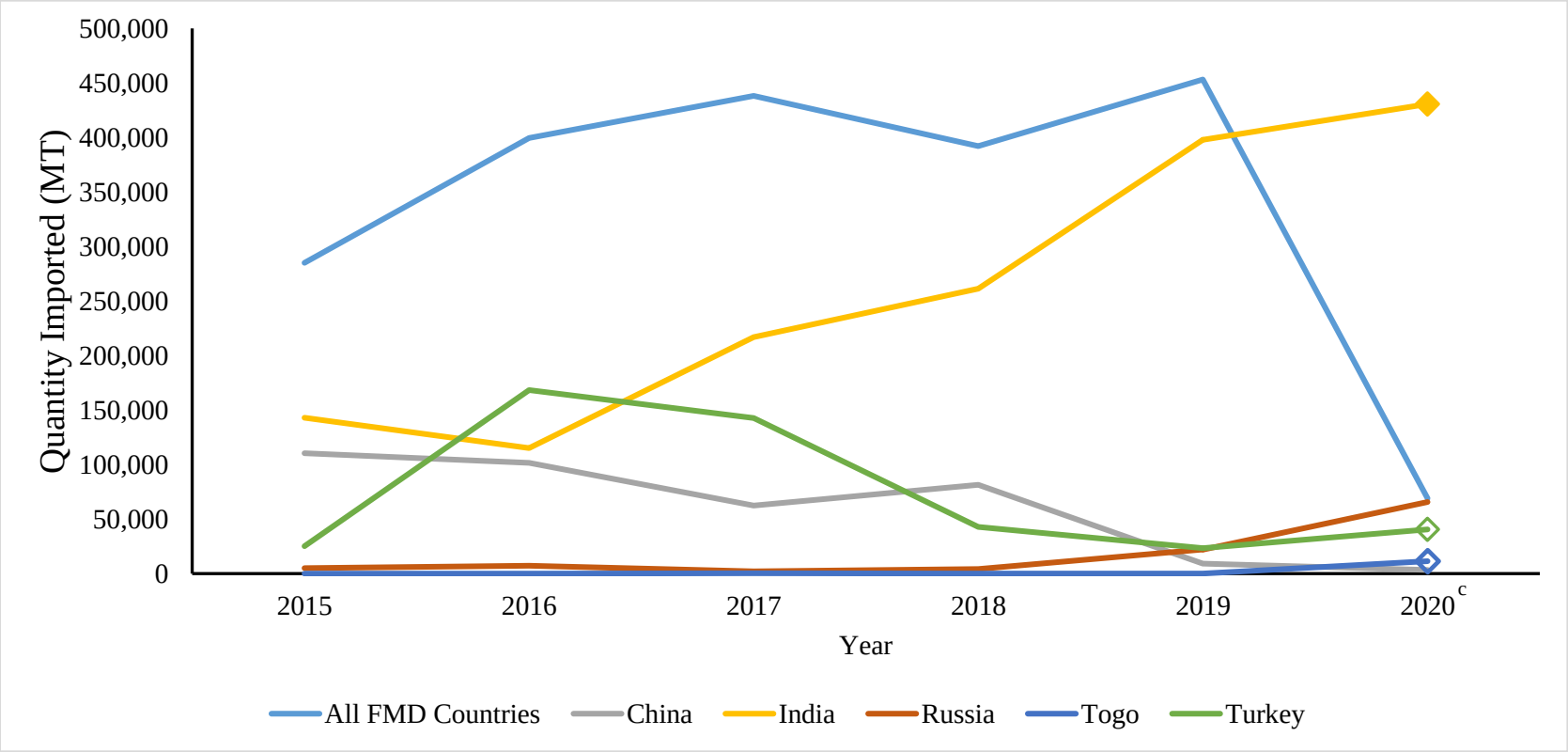
[c] No differentiation provided in figure between countries reporting no ASFV cases and countries with no ASFV data for 2020 in the OIE WAHIS Quantitative Database

Figure 2.3 Imports from countries with classical swine fever virus from 2015 to 2020 and the top 5 exporters of soy to the US by quantity (MT)[a,b]



Dashed lines indicate years that a country did not have CSFV status reported or there were no positive cases.
Diamond markers are the year that a country began to have reported CSFV cases.
Open diamond markers indicate the beginning of years that the country is CSFV-negative or did not have reported cases.
[a] Countries of origin and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.
[b] Classical swine fever status was determined based on presence of cases in a country during each year as reported by the OIE WAHIS Quantitative Data database.
[c]No differentiation provided in figure between countries reporting no CSFV cases and countries with no CSFV data for 2020 in the OIE WAHIS Quantitative Database

Figure 2.4 Imports from countries with foot and mouth disease from 2015 to 2020 and the top 5 exporters of soy to the US by quantity (MT)[a,b]



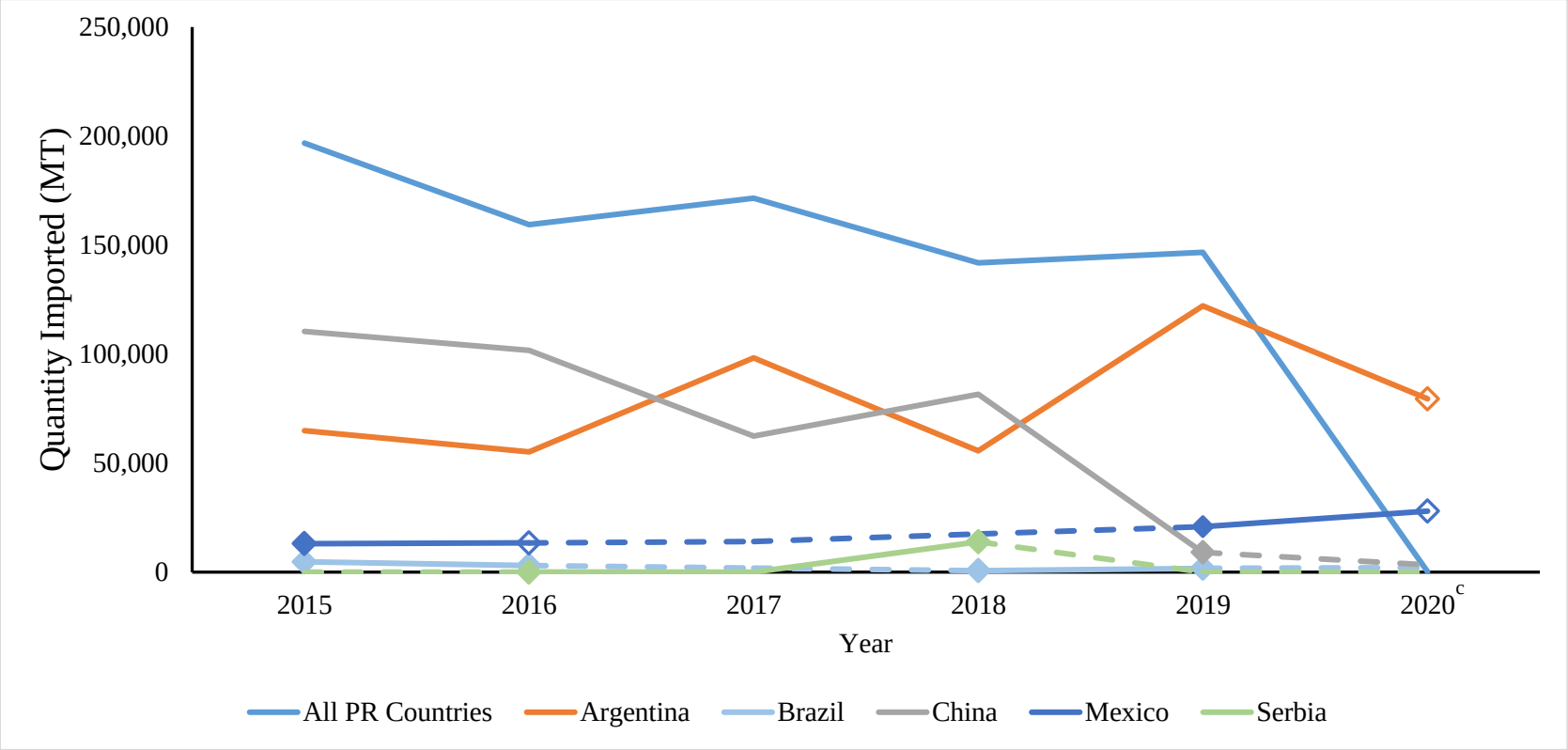
Open diamond markers indicate the beginning of years that the country is FMDV-negative or did not have reported cases.

[a] Countries of origin and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.

[b] Foot and mouth disease status was determined based on presence of cases in a country during each year as reported by the OIE WAHIS Quantitative Data database.

[c] No differentiation provided in figure between countries reporting no FMDV cases and countries with no FMDV data for 2020 in the OIE WAHIS Quantitative Database

Figure 2.5 Imports from countries with pseudorabies from 2015 to 2020 and the top 5 exporters of soy to the US by quantity (MT)[a,b]



Dashed lines indicate years that a country did not have pseudorabies status reported or there were no positive cases.
Diamond markers are the year that a country began to have reported pseudorabies cases.
Open diamond markers indicate the beginning of years that the country is pseudorabies-negative or did not have reported cases.
[a] Countries of origin and quantities (MT) of soy imports were obtained from the United States International Trade and Tariff Database.
[b] Pseudorabies status was determined based on presence of Aujeszky's disease cases in a country during each year as reported by the OIE WAHIS Quantitative Data database.
[c]No differentiation provided in figure between countries reporting no pseudorabies cases and countries with no pseudorabies data for 2020 in the OIE WAHIS Quantitative Database

Chapter 3 - Effect of benzoic acid and commercial essential oil blends on viral load in swine feed and vitamin premix inoculated with PEDV, PRRSV, and SVA viruses

ABSTRACT

Feed has been shown to have the ability to harbor viable viruses of interest for swine producers over an extended period. The use of mitigants and kill steps have been investigated with variable results. This study aimed to investigate the use of benzoic acid (BA) and an essential oil blend (EO) to mitigate the presence of porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome virus (PRRSV), and Senecavirus A (SVA) in a complete diet (Exp. 1) and PEDV in a vitamin premix (Exp. 2). For Exp. 1, four treatments consisting of 0.5% BA, 0.5% BA and 200 ppm EO, 0.3% BA and 120 ppm EO, or 0.25% BA and 100 ppm EO were used in the complete feed in addition to a positive control with no feed additive to test the mitigant's effectiveness on reducing PEDV, PRRSV, and SVA detection via PCR. For Exp. 2, a vitamin premix with 2.68% EO and the same premix with 2.68% limestone were tested to determine the effects on PEDV RNA detection. The inoculated feed or premix was stored for up to 15 d with sampling points at 2, 5, and 15 d post-inoculation (dpi). Samples were analyzed using a triplex qRT-PCR to detect changes in RNA quantities for all three viruses. For Exp. 1, PEDV and SVA had a treatment × day interaction ($P \leq 0.008$). At 2 and 5 dpi, more PEDV RNA was detected in the 0.5% BA treatment than the control, as indicated by a lower Ct value ($P < 0.05$). For SVA, there was an increase in detectible RNA at 15 dpi than the positive controls at the same time point. No interaction was demonstrated for PRRSV, but day

independently reduced viral load rapidly from d 2 to 5 and more slowly until d 15 (quadratic, P = 0.038). The use of the EO in the premix did not demonstrate a treatment × day interaction (P = 0.962), an effect of treatment (P = 0.066), or degradation over time (P > 0.279). In conclusion, the use of a BA and/or EO mitigant in this model did not provide evidence for viral mitigation through the addition of these feed additives in either feed or premix, but viral load was reduced in the feed matrix over time.

INTRODUCTION

Several viruses of interest to swine producers have been shown to survive in feed or feed ingredients over time (Dee et al., 2016; Dee et al., 2018). Contaminated feedstuffs have been linked to the porcine epidemic diarrhea virus (PEDV) outbreak in North American swine herds (Pasick et al., 2014; Bowman et al., 2015). This contaminated feed can cause disease in pigs, therefore finding ways to reduce viral load would be beneficial in the feed manufacturing process (Dee et al., 2014; Pillatzki et al., 2015; Schumacher et al., 2016). Several different methods have been investigated for both point-in-time mitigation as well as extended protection in the form of feed additives. Thermal processing and irradiation have both been shown to be effective at reducing PEDV in feed, but they do not provide any protection if virus is reintroduced to the feed during a later handling step (Trudeau et al., 2016; Cochrane et al., 2017). Feed additives, such as medium-chain fatty acids (MCFAs), acidifiers, formaldehyde, or essential oils (EOs) have also been studied and demonstrated to be beneficial at reducing the amount of detectible virus in feed and feedstuffs (Dee et al., 2015; Gebhardt et al., 2019; Lerner et al., 2020). Many studies have focused on complete feed, grain products, or animal byproducts when investigating viral contamination and subsequent mitigation, but few have looked specifically at vitamins or premixes in their design. Of the studies that investigated these ingredients, the products used

were individual vitamins and not a combined vitamin premix (Dee et al., 2016; Dee et al., 2018; Caserta et al., 2022). The objective of this experiment was to investigate the use of benzoic acid (BA) and a commercially available EO blend to mitigate the presence of PEDV, porcine reproductive and respiratory syndrome virus (PRRSV), and Senecavirus A (SVA) in a complete diet and PEDV in a vitamin premix. To accomplish this objective, two experiments were conducted: 1) to determine the impact of varying levels of BA and EO inclusion in swine gestation feed on detectible PEDV, PRRSV, and SVA using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and 2) to determine the effect of EO inclusion with a vitamin premix on stability of PEDV over time.

MATERIALS AND METHODS

General

Experiment 1: Feed. Treatment structure for the first experiment was arranged as 5 × 3 factorial with five treatments and three timepoints in a complete diet (FEED). The treatments consist of no feed additive for the positive control, addition of benzoic acid (BA; 0.50%, DSM Nutritional Products Inc., Parsippany, NJ), and addition of an essential oil blend (EO; DSM Nutritional Products Inc., Parsippany, NJ) in addition to the BA (0.50% BA and 200 ppm EO, 0.30% BA and 120 ppm EO, or 0.25% BA and 100 ppm EO). The second factor was day of analysis (2, 5, and 15 d post-inoculation; dpi). The diet used was a complete swine gestation diet (Table 1) that was corn and soybean meal based with no specialty ingredients (whey, animal plasma protein, or fish products). There was also a control that contained feed, but no feed additive or virus. There were three replications per treatment.

Experiment 2: Vitamin Premix. Treatment structure for the second experiment was arranged as a 2 × 3 factorial with two treatments and three timepoints. The treatments consisted

of a control containing 2.86% limestone in the vitamin premix used in Exp. 1 or a mitigant treatment of 2.86% EO included at the expense of the limestone. The timepoint factor was day of analysis (2, 5, or 15 dpi). The premix used in Exp. 2 was the same vitamin premix included in the gestation diet used in Exp. 1. There were three replications per treatment.

Feed Preparation and Chemical Treatment

Treatments were applied to 100 g of FEED (Exp. 1) or premix (Exp. 2) and placed in a 1 quart wide-mouth mason jar and mixed with a benchtop mason jar mixer (Central Machine Shop, Purdue University, West Lafayette, IN) as previously described (Lerner et al., 2020; Nichols et al., 2020). Feed or premix was mixed for 15 minutes with 10 hex nuts to provide adequate agitation. After mixing, three aliquots of 17.5 g of feed matrix or premix from each treatment was placed into three polyethylene bottles per timepoint (250 mL Nalgene, square wide-mouth high-density polyethylene; Thermo Fisher Scientific, Waltham, MA).

Viral Inoculation

All treatments except the negative control for the FEED were inoculated with 2.5 mL each of PEDV CO isolate, PRRSV 1.7.4, and SVA SD15-26. The premix EO treatment was inoculated with 2.5 mL of PEDV CO isolate. The three stock viruses contained an initial concentration of 10^6 median tissue culture infectious dose $(TCID)_{50}$/mL.

Inoculation was performed at the Kansas State University College of Veterinary Medicine Virology Laboratory. After addition of virus, each bottle was shaken for 15 s to ensure even distribution of virus through the matrix. The final viral concentration in inoculated bottles was 10^5 $TCID_{50}$/g of matrix.

Real time PCR analysis

Real time PCR was conducted at the Molecular Diagnostics Research and Development laboratory at the Kansas State University Veterinary Diagnostic Laboratory. Separate bottles were analyzed on 2, 5, and 15 dpi. Three bottles of the positive control and three bottles of the negative controls for both the FEED and PREMIX were sampled and analyzed on d 0 to ensure that each matrix was free of viral RNA and to establish a baseline Ct value for the inoculated samples. Samples were stored at room temperature until addition of 100 mL phosphate buffered saline (PBS; pH 7.4 1×, Life Technologies, Grand Island, NY) was added to bottles of 25 g inoculated feed at appropriate time points. After PBS addition, the samples were swirled to ensure even mixing and stored at 4°C for 24 h at which point supernatant was collected and aliquoted for further analysis. The aliquots were stored at -20°C until qRT-PCR was performed.

After collection of d 15 post-inoculation aliquots, qRT-PCR was conducted. Fifty microliters of supernatant from each sample were loaded into a deep-well plate and extracted using a Kingfisher Flex magnetic particle processor (Fisher Scientific, Pittsburg, PA) and the MagMAX-96 Viral RNA Isolation kit (Life Technologies, Grand Island, NY) according to manufacturer's instructions with one modification, reducing the final elution volume to 60 μL. One negative extraction control consisting of all reagents except the sample was included in each extraction. Controls of each stock virus were also included with each extraction. Extracted RNA was frozen at -20°C until assayed by qRT-PCR. Analyzed values represent cycle threshold (Ct) at which virus was detected. Higher Ct values indicate more cycles must proceed before viral genetic material is detected, thus indicating lower quantities of viral genetic material in the sample.

Statistical Analysis

Data were analyzed using PROC GLIMMIX (SAS Institute, Inc., Cary, NC) to determine the interactive and main effects of additive and time on PEDV, PRRSV, or SVA Ct values with sample bottle as the experimental unit. One replicate for the 0.30% BA with 120 ppm EO in FEED was removed from analysis due to having a studentized residual over 4. The Kenward-Roger approach was used to approximate the degrees of freedom. Means were separated with the LSMEANS procedure and the LINES option was used to determine means that differed significantly as determined by an *F* test. Results were considered significant at $P \leq 0.05$. The second experiment used a similar model with the exception of having only PEDV Ct values as the response variable.

RESULTS

Experiment 1

All negative controls at each time point demonstrated no evidence of genetic material for either PEDV, PRRSV, or SVA. There was a significant treatment × day interaction for detection of PEDV in the FEED matrix ($P = 0.008$; Table 2) in which the 0.5% BA treatment had more detectible RNA at 2 and 5 dpi, but had lower amounts of detectible PEDV RNA at 15 dpi compared to the positive control ($P < 0.05$; Table 2). All other treatments had similar amounts of detectible PEDV RNA to the control on all time points. At 5 dpi, the amount of detectable RNA for the 0.5% BA with 200 ppm EO and 0.25% BA with 100 ppm EO treatments did not differ from the 0.5% BA treatment ($P > 0.05$). The three combination treatments did not differ ($P > 0.05$) from the 0.5% BA treatment at 15 dpi for quantities of detectible PEDV RNA. For detectible PRRSV RNA, there was no evidence of a treatment × day interaction ($P = 0.672$). There was a treatment × day interaction for SVA RNA

($P < 0.0001$). At 2 dpi all of the treatments had less detectible ($P < 0.05$) SVA RNA than the positive control. All of the treatments did not differ from control at 5 dpi ($P > 0.05$) and the 0.5% BA treatment had more ($P < 0.05$) detectible SVA RNA than the 0.25% BA with 100 ppm EO treatment. At 15 dpi, the 0.5% BA with 200 ppm EO treatment had similar ($P > 0.05$) amounts detectible SVA RNA to the positive control, with all of the other treatments having more ($P < 0.05$) detectible RNA than the positive control. The 0.5% BA + 200 ppm also had less ($P < 0.05$) detectible SVA RNA than the 0.5% BA treatment. For PRRSV in FEED, there was no evidence of difference due to dietary treatment (Table 3, $P = 0.847$); however, increasing the number of days led to decreased (quadratic, $P = 0.038$; Table 4) amounts of detectible RNA.

Experiment 2

The negative controls for the PREMIX demonstrated no evidence of PEDV RNA at any timepoint. For the PREMIX matrix, there was no evidence of a treatment × day interaction on detectible amounts of PEDV RNA ($P = 0.962$). There was no effect of day observed on detection of PEDV RNA in a premix matrix (linear, $P = 0.279$; quadratic, $P = 0.533$; SEM = 0.48), with 2, 5, and 15 dpi returning Ct values of 33.7, 34.3, and 34.6 respectively. There was a trend ($P = 0.066$, SEM = 0.40) for increased amounts of detectible PEDV in the premix containing EO, with the positive control returning a Ct of 34.8 and the 2.68% EO treatment returning a Ct of 33.6.

DISCUSSION

Research has been conducted to understand the viability and presence of virus in feed to determine how to improve biosecurity practices around swine production and associated feed manufacturing. Investigations into the 2013 PEDV outbreak revealed links to international shipments of ingredients and subsequent spread through the US swine industry (Scott et al.,

2016). Contributing to that theory, Dee et al. (2014) demonstrated the ability of feed contaminated with PEDV to infect naïve pigs. The minimum infectious dose for PRRSV through oral exposure and PEDV in feed are $10^{5.3}$ $TCID_{50}$ and 5.6×10^{1} $TCID_{50}$/g, respectively (Hermann et al., 2005; Schumacher et al., 2016). Caserta et al. (2022) conducted an in vivo experiment with a lowest dose of 10^{5} $TCID_{50}$ of SVA which led to infection of all naïve pigs. Because this dose of SVA was infectious for all the pigs that consumed the inoculated feed, it can be postulated that the lowest infectious dose is less than the lowest infectious dose of PEDV. This ability for viruses to survive and cause clinical disease through feed has led to the development of various methods and products to control viral infectivity in feed matrices.

With increasing time for Exp. 1, PEDV demonstrated significant increases in Ct across all treatments. This ranged between a 4 and 5 Ct increase for each treatment from d 2 to d 15 of the experiment. Interestingly, the 0.5% BA treatment was different from the control at each time point and also had the largest increase in Ct value (27.3 to 33.8) of all PEDV treatments in FEED. While PRRSV did not have a treatment × day interaction, it was quadratically affected by dpi. From 2 to 5 dpi, a 3 Ct increase was demonstrated followed by a 4 Ct increase from 5 to 15 dpi. Of the three viruses, SVA demonstrated the least change in Ct over time, which agrees with previous studies that indicated a longer half-life for SVA than PEDV and other viruses (Dee et al., 2018; Caserta et al., 2022). From 2 to 15 dpi, the increase in Ct ranged from <1 to approximately 1.7 across treatments. Similar stability of SVA RNA was demonstrated in a study conducted by Gebhardt et al. (2019) that also investigated the combination of acidifiers and essential oils blend. In Exp. 2, PEDV in a vitamin premix was also demonstrated to not have a decrease over time in either the control or EO treatment.

The higher Ct values for PEDV in the vitamin premix indicate some effect of the matrix type on the ability to sample the virus, detect it via PCR, or viral survival in the premix. Several viruses, including PEDV, have been demonstrated to survive in vitamins (Dee et al., 2016; Dee et al., 2018). The current study provides information on how the detection of virus in a vitamin premix changes over time both with and without a mitigation treatment as opposed to detection at the end of a timeframe. While premixes are highly processed, components of them may be imported from countries or regions with disease outbreaks and there is still a risk of cross-contamination following initial manufacture.

Vitamin premixes have not been robustly investigated for their ability to harbor infectious virus. A few studies have been conducted with individual vitamins, but results vary based on vitamin type with vitamin D allowing virus to remain infective longer than vitamin A or E (Dee et al., 2016). Vitamins D has also been investigated by Dee (2018) to determine their ability to allow multiple viruses to remain infectious when inoculated into the ingredient. This study demonstrated that SVA, porcine sapelovirus, PEDV, and porcine circovirus type 2 can all remain viable in vitamin D after undergoing a 37 day shipping model. Results in individual vitamins have differed between vitamin suppliers as well (Caserta et al., 2022; Dee et al., 2018). The study conducted by Dee et al. (2018) demonstrated infectivity of SVA after being incubated in vitamin D undergoing transboundary shipping conditions for 37 days, while the Caserta et al. (2022) study was only able to measure infectivity at one day after inoculation of SVA into vitamin D. This highlights that the carriers being used in these vitamin or premix products may be more important than the vitamins themselves, but that has not been investigated at this time.

While the products tested in the current study were inconclusive for viral mitigation, several different strategies have been investigated to reduce the viability of viral contaminants in

feed. Among these mitigants are formaldehyde products, acidifiers, medium chain fatty acids, and essential oils. No strategies are reported as FDA-approved by AAFCO (2021) to reduce viral contamination, but formaldehyde is approved for the control of *Salmonella* species. Formaldehyde has been the most effective against viruses over time and a variety of conditions (Dee et al., 2021; Elijah et al., 2021; Harrison et al., 2021). Medium chain fatty acids (MCFAs) also have demonstrated effectiveness against virus contamination in feed, but not against every virus. When tested against PEDV, PRRSV, and SVA; MCFAs have shown decreases in viral infectivity and presence of genetic material (Lerner et al., 2020; Nichols et al., 2020; Dee et al., 2021; Niederwerder et al., 2021). On the other hand, a study by Jackman et al. (2020) found that adding an MCFA blend to feed did not reduce the infectivity of ASFV contaminated feed at 24 hours post-inoculation. Part of this discrepancy could be due to the different viruses being studied between the experiments and therefore having differing susceptibilities to viral disruption by the MCFA. Acidifiers such as benzoic acid have been investigated to reduce the pH of feed and have been effective against viral infectivity via feed while still having viral RNA detected by PCR (Gebhardt et al., 2019; Dee et al., 2021). Gebhardt et al. (2019) did not demonstrate a decrease in PEDV RNA from 0 to 21 dpi in a feed matrix when compared to the positive control on the same days, which supports the findings from Exp. 1 in the current study, with the 0.5% BA treatment in the feed matrix also not having evidence of a decrease in detected RNA when compared to the control.

The experiment conducted herein failed to demonstrate an effect of benzoic acid and essential oil addition on detected PEDV, PRRSV, or SVA, which was unexpected based on previous research demonstrating the anti-viral potential of both these compounds. A study conducted by Gebhardt et al. (2019) investigated the addition of benzoic acid and essential oils to

feed in order to reduce contamination of PEDV. The study demonstrated no difference in qRT-PCR Ct values between the positive control and the benzoic acid + essential oil treatment until 21 days post-inoculation. Based on this result, it is possible that the current study was not conducted long enough to see positive results from the mitigant addition to the feed, as the last sampling day was 15 days post inoculation. Extending this study out to at least 30 days may be beneficial to demonstrate an effect of the mitigant on detectible levels of virus over time. Another study using similar compounds was conducted to investigate their effects against African swine fever virus by Zhai et al. (2021) demonstrated promising results, with increased Ct values being found in the benzoic acid + essential oil treatment compared to the positive control at just 1 dpi. This could be due to different viral characteristics, diet composition, or single versus co-infection models. A limitation of the model used herein is the lack of infectivity information. While there was not a difference in amounts of RNA detected, this does not mean that the infectivity of the virus in each treatment group is equal. Bioassay is necessary to fully understand the potential of these mitigants in preventing disease and their use in feed biosecurity.

CONCLUSION

In conclusion, results of qRT-PCR analysis of samples from 2 to 15 dpi did not show evidence of treatment with benzoic acid and essential oils on reducing viral RNA in either feed or vitamin premix. More investigation needs to be conducted into the effect of these compounds on the infectivity of the viruses to determine if the treatment is successful in viral inactivation as opposed to viral detection.

Table 3.1 Diet composition (as-fed basis; Exp. 1)

Item:	Swine gestation diet
Ingredient, %	
Corn	78.40
Soybean meal, dehulled, solvent-extracted	17.27
Soybean oil	0.50
Calcium carbonate	1.30
Monocalcium phosphate, 21% P	1.30
Salt	0.50
Trace mineral premix[1]	0.15
Sow add pack[2]	0.25
Vitamin premix[3]	0.25
Phytase[4]	0.08
EO[5]	+/-
BA[6]	+/-
Total	100
Calculated analysis, %	
CP	14.70
Crude fiber	2.20
Ether extract	3.50
Ca	0.91
P	0.61
Available P	0.48

[1]Each kilogram contains 22g Mn, 73g Fe, 73g Zn, 11g Cu, 198 mg I, and 198 mg Se.
[2]Each kilogram contains 4,409 IU vitamin E, 992 mg vitamin B6, 110,229 mg choline, 44.1 mg biotin, and 331 mg folic acid.
[3]Each kilogram contains 1,653,439 IU vitamin A, 661,376 IU vitamin D3, 17,637 IU vitamin E, 13.3 mg vitamin B12, 1,323 mg menadione, 3,307 mg riboflavin, 11,023 mg D-Pantothenic acid, 19,841 mg niacin.
[4]Ronozyme HiPhos (GT) 2700, DSM Nutritional Products Inc., Parsippany, NJ.
[5]EO = essential oils blend (DSM Nutritional Products Inc., Parsippany, NJ) added to complete diet at 200 ppm, 120 ppm, or 100 ppm in appropriate treatments
[6]BA = benzoic acid, (DSM Nutritional Products Inc., Parsippany, NJ) added to complete diet at 0.50%, 0.30% or 0.25% in appropriate treatments

Table 3.2 Effects of treatment and days post-inoculation (dpi) on detection of PEDV, PRRSV, and SVA in complete feed and detection of PEDV in a vitamin premix via qRT-PCR[1,2]

	qRT-PCR Ct, dpi[3]				
	2	5	15	SEM	*P* =[4]
FEED					
PEDV				0.38	0.008
+ Control	28.3^{e}	31.4^{c}	32.7^{b}		
0.5% BA	27.3^{f}	30.3^{d}	33.8^{a}		
0.5% BA, 200 ppm EO	28.7^{e}	30.5^{c,d}	33.6^{a,b}		
0.30% BA, 120 ppm EO	28.6^{e}	31.3^{c}	33.5^{a,b}		
0.25% BA, 100 ppm EO	29.0^{e}	31.1^{c,d}	33.0^{a,b}		
PRRSV				1.54	0.672
+ Control	30.8	34.7	39.5		
0.5% BA	31.3	34.4	39.8		
0.5% BA, 200 ppm EO	31.2	34.1	39.7		
0.30% BA, 120 ppm EO	31.5	34.7	37.0		
0.25% BA, 100 ppm EO	31.5	34.5	36.2		
SVA				0.18	0.0001
+ Control	27.7^{g}	28.7^{b,c,d,e}	29.4^{a}		
0.5% BA	28.2^{f}	28.5^{d,e,f}	28.5^{c,d,e,f}		
0.5% BA, 200 ppm EO	28.4^{d,e,f}	28.3^{e,f}	29.0^{a,b}		
0.30% BA, 120 ppm EO	28.8^{b,c,d}	28.7^{b,c,d,e}	28.8^{b,c,d}		
0.25% BA, 100 ppm EO	28.6^{c,d,e,f}	28.9^{b,c}	28.7^{b,c,d,e}		
PREMIX					
PEDV				0.69	0.962
+ Control	34.4	34.9	35.0		
2.68% EO	33.0	33.7	34.1		

[1]An initial tissue culture (2.5 mL diluted virus inoculum, 10^6 $TCID_{50}$/mL) of PEDV, PRRSV, and SVA was inoculated into 17.5 g of gestation diet (FEED) that was treated with different combinations of benzoic acid (BA; DSM Nutritional Products, Parsippany, NJ), and essential oils blend (EO; DSM Nutritional Products, Parsippany, NJ) with three replicates per treatment per day.

[2]An initial tissue culture (2.5 mL diluted virus inoculum, 10^6 $TCID_{50}$/mL) of PEDV was inoculated into 12.5 g of vitamin premix (PREMIX) with 2.68% EO product or no chemical treatment with three replicates per treatment per day.

[3]Ct = cycle threshold required to detect genetic material. A higher Ct value is indicative of less genetic material present.

[4]Matrix × day post-inoculation interaction. $n = 3$ for each value.

[a,b,c,d,e,f]Means within interaction or main effect lacking a common superscript differ ($P < 0.05$).

Table 3.3 Effect of treatment on detection of PEDV, PRRSV, and SVA in complete feed via qRT-PCR[1,2]

	+ Control	0.5% BA[3]	0.5% BA, 200 ppm EO[4]	0.30% BA, 120 ppm EO	0.25% BA, 100 ppm EO	SEM	*P* =
PEDV	30.8	30.5	30.9	31.1	31.1	0.19	0.135
PRRSV	35.0	35.1	35.0	34.4	34.1	0.78	0.847
SVA	28.6	28.4	28.6	28.8	28.7	0.09	0.062

[1]An initial tissue culture (2.5 mL diluted virus inoculum, 10^6 $TCID_{50}$/mL) of PEDV, PRRSV, and SVA was inoculated into 17.5 g of gestation diet (FEED) that was treated with different combinations of benzoic acid (BA; DSM Nutritional Products, Parsippany, NJ), and essential oils blend (EO; DSM Nutritional Products, Parsippany, NJ) with three replicates per treatment per day.
[2]Ct = cycle threshold required to detect genetic material. A higher Ct value is indicative of less genetic material present.
[3]BA = benzoic acid (DSM Nutritional products, Parsippany, NJ)
[4]EO = essential oils blend (DSM Nutritional products, Parsippany, NJ)

Table 3.4 Effect of days-post-inoculation (dpi) on detection of PEDV, PRRSV, and SVA in complete feed via qRT-PCR[1,2]

	Day				Contrasts	
	2	5	15	SEM	Linear	Quadratic
PEDV	28.4	30.9	33.3	0.14	< 0.0001	< 0.0001
PRRSV	31.3	34.5	38.4	0.59	< 0.0001	0.038
SVA	28.3	28.6	28.9	0.07	< 0.0001	0.131

[1]An initial tissue culture (2.5 mL diluted virus inoculum, 10^6 $TCID_{50}$/mL) of PEDV, PRRSV, and SVA was inoculated into 17.5 g of gestation diet (FEED) that was treated with different combinations of benzoic acid (BA; DSM Nutritional Products, Parsippany, NJ), and essential oils blend (EO; DSM Nutritional Products, Parsippany, NJ) with three replicates per treatment per day.

[2]Ct = cycle threshold required to detect genetic material. A higher Ct value is indicative of less genetic material present.

Chapter 4 - Viral isolation of Porcine Reproductive and Respiratory Syndrome virus from feed ingredients and complete feed with subsequent qRT-PCR analysis

ABSTRACT

Rapid, affordable detection of virus in feed relies on polymerase chain reaction (PCR) and the resulting cycle threshold (Ct) values. The Ct values only indicate if the genetic material of the virus is present, not likelihood of infection. This study aimed to use viral isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR) to determine infectivity and genetic material stability of PRRSV and when incubated in soybean meal (SBM), dried distiller's grains with solubles (DDGS), or a corn-soy based swine feed (FEED) at three different temperatures over three days. Solvent-extracted soybean meal (SBM), dried distiller's grains with solubles (DDGS), complete swine feed (FEED), or media (DMEM) were inoculated with $10^{5.4}$ $TCID_{50}$/mL of type 2 PRRSV strain 129 and incubated at 4°C, 23°C, or 37°C. Samples were taken at one, 24, 48, and 72 hours post-inoculation and suspended in DMEM before centrifugation and filtration. Supernatant was then titrated and used to inoculate confluent MARC-145 cells and $TCID_{50}$/mL was determined. Each supernatant sample underwent RNA extraction and was used for qRT-PCR to determine the change in detectible virus across matrix type, temperature, and time. An interaction (P = 0.0278) was observed for matrix × temperature × hour for live virus detected in the VI model. At 4°C, infectivity was greatest in DMEM, with SBM intermediate and DDGS and FEED the least infectious. All feedstuffs had decreased viral infectivity compared to the DMEM at 23°C over time, with SBM maintaining a higher

infectivity than DDGS and FEED at 1hr and 24hr post-inoculation. At 37°C, the DMEM sustained greater infectivity than the three feedstuffs 1hr post-inoculation with decreasing infectivity until 48hr with no further decrease. The SBM, DDGS, and FEED demonstrated less infectious PRRSV than the DMEM from 1hr to 24hr post-inoculation. Only matrix type influenced the quantity of viral RNA detected using qRT-PCR ($P = 0.0319$). The viral control had more viral RNA detected than DDGS with SBM and FEED being intermediate. This VI model demonstrates the ability of SBM, DDGS, and FEED to harbor infectious virus, and storage time and temperature influence virus inactivation. The interactive response demonstrates that further research is needed to determine if the decreased infectivity is from PRRSV being inactivated or if the virions are bound to organic material, making them undetectable which would require future bioassay to confirm infectivity potential.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) can cause decreased productivity and significant economic impacts (Neumann et al., 2005; Nieuwenhuis et al., 2012). Porcine reproductive and respiratory syndrome has become a seasonal challenge for the US swine industry with many systems utilizing filtered barns to prevent airborne introduction (Dee et al., 2010; Spronk et al., 2010; Yeske, 2012). The disease can cause up to a 23% decrease in production of wean pigs and increases in abortions and repeat breeding in sows, leading to a need to understand infectivity and transmission to prevent introduction to farms (Valdes-Donoso et al., 2018).

Transmission of PRRSV is typically via aerosol, but it also can survive in environment, manure, or feed. The half-life for infectious PRRSV can vary based on the matrix type, temperature, and humidity with higher temperatures and humidity decreasing the half-life

(Hermann et al., 2007; Linhares et al., 2012). While the half-life is shorter than other matrix types, there is evidence that PRRSV can survive in feedstuffs, which could be a concern for introduction into a farm (Dee et al., 2018). Reliable RNA detection is a challenge when dealing with non-clinical samples such as feed or environmental sampling. This is largely due to commercial PRRSV PCR testing being originally developed for clinical samples such as tissues or serum.

The most common method for detecting PRRSV or any virus in animal feed is completed by using PCR which uses non-standardized extraction methods. This only provides information on if the viral genome is present, not if it is infectious. The most reliable method for determining infectivity of contaminated feed is done using bioassay which is expensive and time consuming. A benchtop model using MARC-145 cells to determine infectivity would allow for faster, more cost-effective identification of infectious PRRSV in animal feed. Therefore, this study aimed to use viral isolation and qRT-PCR to determine infectivity and genetic material stability of PRRSV and when incubated in soybean meal (SBM), dried distiller's grains with solubles (DDGS), or a corn-soy based swine feed (FEED) at three different temperatures over three days.

METHODS

General

Treatment structure for this experiment was a split plot design with time nested within matrix × temperature. Three different feed matrices were used (soybean meal, DDGS, and a corn-soy swine diet with 0.5% fat) and inoculated with type 2 PRRSV strain 129 with a concentration of $10^{5.4}$ $TCID_{50}$/ml for a final concentration of $10^{4.4}$ $TCID_{50}$/g of matrix. Each matrix was incubated at three different temperatures (4, 23, and 37°C) for a total of nine treatments with samples taken at 1, 24, 48, and 72 hours post-inoculation. Each treatment was

replicated in duplicate. Samples were suspended in Dulbecco's modified eagle medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% bovine calf serum (HyClone, Cytiva, Marlborough, MA, USA), ultraglutamine (2mM/L; Lonza, Basel, Switzerland), anti-anti (100,000 units/L of penicillin, 100 mg/L of streptomycin, and 0.25 mg/L of Fungizone; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and Fungizone (2.5 mg/L of amphotericin B and 2.05 mg/L of sodium deoxycholate; HyClone, Cytiva, Marlborough, MA, USA) prior to centrifugation and removal of supernatant. The supernatant was syringe filtered and serially diluted from 10^{-1} to 10^{-6}. Two aliquots of supernatant were frozen at -80°C for quantitative reverse transcription polymerase chain reaction (qRT-PCR) or future analysis. Viral isolation was conducted by applying the undiluted and diluted supernatant in triplicate to confluent MARC-145 cells in 96-well plates. The inoculated cells were incubated at 37°C for 48 hours before being fixed and frozen at -20°C until staining. The viral isolation plates were stained using SDOW17-A conjugated antibodies and anti-mouse FITC conjugated antibodies. The $TCID_{50}$/ml of each supernatant sample at each time point for each treatment was recorded. One aliquot of each sample was thawed and had 200 μm removed for RNA extraction. The viral RNA was isolated from each sample using the Quick-RNA™ Viral Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. One negative extraction control consisting of all reagents except the sample was included with each extraction. Extracted RNA was frozen at -80°C until assayed by qRT-PCR. Each qRT-PCR run included four dilutions of the stock virus to create a standard curve of PRRSV RNA and allow for calculation of viral RNA in experimental samples. Analyzed values represent $\log_{10}$ of detected virions.

Statistical analysis

Viral isolation data were analyzed to determine the main effects of matrix type, temperature, and time in relation to amount of infectious PRRSV detected with sample as the experimental unit and tube as a random effect with a compound-symmetry structure to account for repeated measurement on each tube over time. Two replicates were conducted for each matrix-temperature combination. The cell culture media + PRRSV was aliquoted three times at each time point to be run alongside each matrix time, so aliquot number nested within tube was include in the model as a random intercept. One subsample for the cell culture media + PRRSV after 24 hr at 4°C was removed from analysis due to a studentized residual over 6.

The qRT-PCR data were analyzed to determine the main effects of matrix type, temperature, and time in relation to amount of PRRSV RNA detected with sample as the experimental unit and tube as a random effect with compound-symmetry structure to account for repeated measurements on each tube over time. Two replicates were conducted for each matrix-temperature combination. All models were fit using PROC GLIMMIX (SAS Institute, Inc., Cary, NC). The Kenward-Roger approach was used to approximate the degrees of freedom. Means were separated with the LSMEANS procedure and the LINES option was used to determine means that differed significantly as determined by an *F* test. Results were considered significant at $P \leq 0.05$.

RESULTS

There was a matrix × temperature × hour interaction ($P = 0.0278$) for the infectivity of PRRSV in the viral isolation assay (Table 1). When inoculated into DMEM, PRRSV maintained elevated concentrations of infectious virus from 1 hr to 72 hr (3.61 to 3.72 $TCID_{50}$/ml, respectively) at 4°C. The concentration of infectious PRRSV was similar ($P > 0.05$) from 1 to 72

hpi (2.94 to 2.22 $TCID_{50}$/mL, respectively) at 23°C. However, at 72 hpi, DMEM samples at 23°C had decreased ($P < 0.05$) concentrations of infectious virus when compared to 4°C (2.22 and 3.72 $TCID_{50}$/ml, respectively). Finally, when incubated in DMEM at 37°C, the concentration of infectious PRRSV decreased ($P < 0.05$) over time from 3.5 $TCID_{50}$ at 1 hpi, to 1.94 $TCID_{50}$/ml at 24 hpi, and 0.78 and 0.42 $TCID_{50}$/ml at 48 and 72 hpi, respectively. At each timepoint after 1 hpi, the sample incubated at 37°C contained less infectious PRRSV than when incubated at 4 or 27°C.

The contaminated SBM was hag a high level of infectious virus at all time points when incubated at 4°C (between 2.50 and 3.00 $TCID_{50}$/mL). In comparison to 4°C, PRRSV had decreased ($P < 0.05$) concentrations of infectious virus at 1 and 24 hpi (1.67 and 1.25 $TCID_{50}$/mL, respectively) at 23°C. No virus was detected at 48 hpi, and low amounts of infectious virus (0.83 TCID50/mL) was detected at 72 hpi. Finally, the concentration of infectious PRRSV in SBM decreased ($P < 0.05$) dramatically when incubated at 37°C. Infectious virus was only detected at 1 hpi (1.00 $TCID_{50}$/mL). Porcine respiratory and reproductive syndrome virus contaminated SBM demonstrated similar ($P > 0.05$) concentrations of infectious virus to the contaminated DMEM at 4°C until 72 hpi. At this time point and temperature, the concentration of infectious PRRSV in SBM decreased ($P < 0.05$) compared to the DMEM. The contaminated SBM incubated at 23°C had decreased ($P < 0.05$) concentrations of infectious virus at all timepoints than the DMEM at the same temperature, and no infectious virus was detected at 48 hpi. The PRRSV contaminated SBM also demonstrated decreased ($P < 0.05$) concentrations of infectious virus compared to the DMEM when incubated at 37°C, with low infectivity at 1 hpi (1.00 $TCID_{50}$/mL) and no infectivity detected after that time.

At 4°C, the contaminated DDGS started with moderate levels of infectious PRRSV at 1 hpi (2.33 $TCID_{50}$/mL) and decreased ($P < 0.05$) until 72 hpi (0.92 $TCID_{50}$/mL). The PRRSV demonstrated low levels of infectious virus after incubation at 23°C. From 1 to 48 hpi, the concentration of infectious virus ranged from 0.42 to 0.58 $TCID_{50}$/mL, and no infectivity was detected at 72 hpi. No infective PRRSV was detected at any time point when contaminated DDGS were incubated at 37°C. When incubated at 4°C, PRRSV contaminated DDGS contained decreased ($P < 0.05$) concentrations of infectious virus at each time point compared to the DMEM at the same temperature. The difference in infectivity between the DDGS and DMEM is even wider at 23°C with 1 hpi having an infectivity of 0.42 and 2.94 $TCID_{50}$/mL for the DDGS and DMEM respectively. This difference in quantity of infectious virus became even larger with decreasing ($P < 0.05$) to no infectivity detected at 72 hpi in the DDGS and 2.22 $TCID_{50}$/mL in the DMEM. At 37°C, no infectious virus was detected in the DDGS, while the DMEM still had measurable infectious PRRSV.

Finally, the PRRSV contaminated FEED demonstrated low levels of infectious virus over time at each temperature. At 4°C, low quantities of infectious virus was detected at 1, 24, and 72 hpi (0.75, 0.42, and 0.42 $TCID_{50}$/ml, respectively), with no infectivity detected in either sample at 48 hpi. When incubated at 23°C, contaminated FEED maintained similar ($P > 0.05$) levels of infectious virus over time with 1 and 24 hpi both having $TCID_{50}$/mL of 1.00 before decreasing ($P < 0.05$) to 0.00 at 48 and 72 hpi. Finally, 1 hpi was the only time point with a sample with detectible infectivity (0.58 $TCID_{50}$/ml) for contaminated FEED incubated at 37°C. The FEED had much less ($P < 0.05$) infectious virus detected at all time and temperature combinations than was detected in the DMEM at the same conditions.

When the supernatant samples were analyzed by qRT-PCR, no significant interaction ($P = 0.4793$) was observed for matrix × temperature × hour viral copies (Table 2). However, a main effect of matrix type was significant ($P = 0.0319$) for viral copy amount from qRT-PCR (Table 3). The contaminated DMEM had the highest recovery of viral copies with $10^{5.26}$ viral copies/ml and DDGS had the lowest with $10^{2.48}$ viral copies/ml, with SBM and FEED being intermediate ($10^{4.18}$ and $10^{3.86}$ viral copies/ml, respectively).

DISCUSSION

In this study, there were some common trends that could be observed between several matrix types over certain temperatures. When incubated at 4°C, the DMEM, SBM, and FEED all maintained the quantity of infectious PRRSV across each time point. The DMEM allowed the virus to maintain the highest concentration of infectious PRRSV, followed closely by the SBM, while the FEED had a very low level of infectious virus across the whole time period while refrigerated. Both the DDGS and FEED had very low or no infectious virus detected similar to each other across the whole study period at 23°C and 37°C. The SBM and DDGS both demonstrated decreased quantity of infectious PRRSV with increasing temperature. A similar decrease in infectious virus was not shown in the DMEM until 37°C.

The effect of matrix type can be compared to research by other teams and their investigations into viral stability in cell cultures and environmental samples. Several studies have investigated the stability of PRRSV in cell culture media over time and temperatures. Bloemraad et al. (1994) demonstrated a decrease in the half-life of the virus from 140h at 4°C to 20 h at 21°C, and just 3 h at 37°C. While the current study did not calculate half-life, the loss of infectivity in the DMEM at each temperature was consistent with these previously reported decreases in virus stability. Jacobs et al. (2010) also illustrated an exponential decrease in the

half-life of PRRSV in cell culture media with increasing temperature. Dee et al. (2018) demonstrated this variability between different matrix types. In that study, some viruses, including PRRSV, were found to be infectious in some feedstuffs 30 days after inoculation while no infectivity was detected in other products. The aforementioned study included SBM, DDGS, and a complete diet as matrices, and both the SBM and DDGS did not demonstrate evidence of infectious virus presence when evaluated using VI, but did demonstrate infectivity when evaluated using bioassay. This is particularly interesting for the current study as several sampling points for the DDGS were did not contain detectible PRRSV via VI, but may still have infectivity potential when fed. The half-life estimates for individual viruses also changed based on the matrix type in the same study (Dee et al., 2019). Research by Linhares et al. (2012) demonstrated that PRRSV in manure had a longer infectious half-life than controls incubated in culture media at the same temperature. Because of the substantial difference in moisture between feces and feed and the increased presence of fats or other molecules that could impact the viral envelope, it would be reasonable to expect a greater decrease of viral infectivity in feed and feedstuffs compared to other environmental samples due to the presence of other reactive compounds, fat, and the complexity of the matrix. The current study has demonstrated that the decrease in infectivity can change based on which ingredient is being tested as well as the difference between single ingredients and complete feeds.

While FEED had the lowest infectivity over time and temperature, DDGS had the lowest recovery of viral genetic material. This could indicate that components of DDGS are able to bind the virus into the matrix and prevent the virions from suspending into the supernatant. One explanation is that residual ethanol in the DDGS could interact with the viral envelope and disrupt the virion, leading to degradation of the RNA. Of the three matrices, DDGS are the most

acidic and the lower pH could have created an issue with viral detection. Another explanation would be that fatty acids in the DDGS are disrupting the viral envelope and aiding in denaturing the RNA. The anti-viral effects of fatty acids, particularly medium-chain fatty acids (MCFAs), and monoglycerides have been demonstrated previously when supplied via milk or feed additives, so the ability of DDGS to disrupt the viral envelope is a possibility (Thormar et al., 1987; Cochrane et al., 2020; Jackman et al., 2020). Schmidt et al. (1979) found a lipid-glycoprotein complex when palmitate ($C_{16}H_{32}O_2$) was added to Sindbis virus, an enveloped Alphavirus. This lipid-glycoprotein interaction indicated that the anti-viral effect of this fatty acid on Sindbis virus prevented the virus from budding from the host cell. Kohn et al. (1980) demonstrated a decrease in viral infectivity when free linoleic acid was added to a cell culture at the same time as Sindbis virus and electron microscopy indicated that the viral envelope may be destroyed by the fatty acid. In the same study, non-enveloped viruses did not demonstrate the same reduction in infectivity. Hilmarsson et al. (2005) also demonstrated that lowering the pH of the environment increased the anti-viral effect of fatty acids. This is particularly relevant because the U.S. Grains Council (2012) suggests the pH of DDGS ranges from 3.6 to 5, which may explain the decreased detection of PRRSV genetic material in the study conducted herein.

The relation between PCR results and VI results is not easy to describe. Each of these tests measure a different aspect of the virus in question. While PCR has the sensitivity to measure even one virion in a mL of sample, it only indicates that the RNA is present and not the viability of that virion. Infectivity assays, like VI, are required to determine if that virus is able to bind to its target, replicate in the cell, and proliferate. Several factors can impact the ability for a virus to do any of these necessary steps for infection. One such factor may be that that the proteins necessary for binding to the host cell could be blocked or denatured. Another method to

prevent infectivity may be that host cell membrane instability would prevent the proliferation of the virus by not allowing it to exit the host cell with its envelope. There are multiple reasons why a sample of virus may not be measured to be infectious, but could still have its genetic material intact to be detected by PCR. Another limitation of using a VI assay is the limit of detection for such a model. While more expensive, bioassay could be useful to confirm negative infectivity results from a VI model because bioassay is a more sensitive test.

The fact that the DDGS generally had a higher infectivity than the FEED adds complexity to the results of the study herein. This would indicate that the DDGS had more active, replicating PRRSV per mL compared to the FEED samples; however, the DDGS has a higher Ct value than the FEED, indicating less PRRSV RNA per mL in the DDGS compared to the FEED. Jacobs et al. (2010) noted that qRT-PCR did not indicate any difference in detection of PRRSV RNA over time or temperature, while an infectivity assay demonstrated a decrease in infectivity over time and as temperature increased. A similar effect was seen in the present study, with the viral isolation assay demonstrating a matrix × temperature × time effect on the infectivity of PRRSV, while the qRT-PCR data only indicate an impact of time on the detection of the virus. More investigation is warranted to understand the different responses between VI And qRT-PCR of feed matrices inoculated with PRRSV.

CONCLUSION

In conclusion, SBM and DDGS are both capable of harboring infectious PRRSV for up to and potentially beyond 3 days at cold temperatures, but this is less likely in FEED. The reduced infectivity over time is more drastic as temperatures increase. When comparing the qRT-PCR analysis of being intermediate. The low recovery of PRRSV RNA from contaminated DDGS warrants more investigation into sampling procedures to maximize detection.

Table 4.1 Effects of matrix type, hour post-inoculation (hpi), and storage temperature on infectivity of PRRSV via viral isolation[1]

Matrix/ hour		4°C		23°C		37°C			
		Log_{10} $TCID_{50}$/ml	Proportion Positive	Log_{10} $TCID_{50}$/ml	Proportion Positive	Log_{10} $TCID_{50}$/ml	Proportion Positive	SEM	P = [2]
DMEM[3]								0.3075	0.0278
	1	$3.61^{a,b}$	2/2	$2.94^{a,b,c,d}$	2/2	$3.50^{a,b}$	2/2		
	24	3.75^{a}	2/2	$3.00^{a,b,c,d}$	2/2	$1.94^{e,f,g,h,i,j}$	2/2		
	48	$3.39^{a,b,c}$	2/2	$2.67^{b,c,d,e}$	2/2	$0.78^{k,l,m,n}$	1/2		
	72	3.72^{a}	2/2	$2.22^{d,e,f,g,h,i}$	2/2	$0.42^{m,n}$	1/2		
SBM[4]								0.3773	
	1	$2.83^{a,b,c,d,e}$	2/2	$1.67^{f,g,h,i,j,k}$	2/2	$1.00^{j,k,l,m,n}$	2/2		
	24	$3.00^{a,b,c,d}$	2/2	$1.25^{i,j,k,l,m}$	1/2	0.00^{n}	0/2		
	48	$2.67^{b,c,d,e,f}$	2/2	0.00^{n}	0/2	0.00^{n}	0/2		
	72	$2.50^{c,d,e,f,g}$	2/2	$0.83^{k,l,m,n}$	2/2	0.00^{n}	0/2		
DDGS[5]								0.3773	
	1	$2.33^{d,e,f,g,h}$	2/2	$0.42^{m,n}$	1/2	0.00^{n}	0/2		
	24	$1.50^{g,h,i,j,k,l}$	2/2	$0.58^{l,m,n}$	1/2	0.00^{n}	0/2		
	48	$1.33^{h,i,j,k,l,m}$	2/2	$0.42^{m,n}$	1/2	0.00^{n}	0/2		
	72	$0.92^{k,l,m,n}$	1/2	0.00^{n}	0/2	0.00^{n}	0/2		
FEED[6]								0.3773	
	1	$0.75^{k,l,m,n}$	1/2	$1.00^{j,k,l,m,n}$	2/2	$0.58^{l,m,n}$	1/2		
	24	$0.42^{m,n}$	1/2	$1.00^{j,k,l,m,n}$	2/2	0.00^{n}	0/2		
	48	0.00^{n}	0/2	0.00^{n}	0/2	0.00^{n}	0/2		
	72	$0.42^{m,n}$	1/2	0.00^{n}	0/2	0.00^{n}	0/2		

[1]An initial tissue culture (1 mL diluted virus inoculum, $10^{5.4}$ $TCID_{50}$/mL) of PRRSV 129 was inoculated into 10 g of soybean meal (SBM), dried distiller's grains with solubles (DDGS), or gestation diet (FEED) and stored at 4, 23, or 37°C for 72 hours with sampling points at 1, 24, 48, and 72 hpi. Samples were suspended in cell culture media prior to centrifugation and titration of the supernatant over MARC 145 cells for viral isolation.

[2]Matrix × hour × temperature interaction. *n* = 2
[3]DMEM = Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)
[4]SBM = soybean meal
[5]DDGS = Dried distiller's grain with solubles
[6]FEED = complete swine gestation diet
[a,b,c,d,e,f,g,h,I,j,k,l,m,n]Means lacking a common superscript differ ($P < 0.05$).

Table 4.2 Interactive effects of matrix × temp × hour on log_{10} genomic copies/ml of sample supernatant as detected by qRT-PCR[1]

Matrix/ hour	4°C		23°C		37°C			
	Log_{10} genomic copies/ml	Proportion PCR Positive	Log_{10} genomic copies/ml	Proportion PCR Positive	Log_{10} genomic copies/ml	Proportion PCR Positive	SEM	P = [2]
DMEM[3]							1.408	0.4793
1	5.40	2/2	3.76	2/2	5.77	2/2		
24	5.68	2/2	5.47	2/2	5.82	2/2		
48	5.44	2/2	5.93	2/2	5.58	2/2		
72	5.69	2/2	5.90	2/2	2.63	1/2		
SBM[4]								
1	5.63	2/2	2.88	1/2	2.72	1/2		
24	5.72	2/2	5.53	2/2	2.66	1/2		
48	5.59	2/2	5.63	2/2	2.73	1/2		
72	2.84	1/2	5.68	2/2	2.60	1/2		
DDGS[5]								
1	4.08	2/2	4.66	2/2	2.31	1/2		
24	1.85	1/2	3.52	2/2	0.00	0/2		
48	4.02	2/2	4.16	2/2	1.77	1/2		
72	0.00	0/2	2.00	1/2	1.38	1/2		
FEED[6]								
1	2.25	1/2	4.26	2/2	2.49	1/2		
24	4.70	2/2	1.99	1/2	4.07	2/2		
48	4.71	2/2	4.89	2/2	4.05	2/2		
72	4.46	2/2	4.68	2/2	3.72	2/2		

[1]An initial tissue culture (1 mL diluted virus inoculum, $10^{5.4}$ $TCID_{50}$/mL) of PRRSV 129 was inoculated into 10 g of soybean meal (SBM), dried distiller's grains with solubles (DDGS), or gestation diet (FEED) and stored at 4, 23, or 37°C for 72 hours with sampling

points at 1, 24, 48, and 72 hpi. Samples were suspended in cell culture media prior to centrifugation and qRT-PCR analysis of supernatant

[2]Matrix × hour × temperature interaction. *n* = 2

[3]DMEM = Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)

[4]SBM = soybean meal

[5]DDGS = Dried distiller's grain with solubles

[6]FEED = complete swine gestation diet

Table 4.3 Main effect of matrix type on log_{10} genomic copies/ml of sample supernatant as detected by qRT-PCR[1]

Matrix	Log_{10} genomic copies/ml	Proportion PCR Positive	SEM	*P* =
DMEM[2]	5.26[a]	23/24	0.564	0.0319
SBM[3]	4.18[a,b]	18/24		
DDGS[4]	2.48[b]	18/24		
FEED[5]	3.86[a,b]	21/24		

[1]An initial tissue culture (1 mL diluted virus inoculum, $10^{5.4}$ $TCID_{50}$/mL) of PRRSV 129 was inoculated into 10 g of soybean meal (SBM), dried distiller's grains with solubles (DDGS), or gestation diet (FEED) and stored at 4, 23, or 37°C for 72 hours with sampling points at 1, 24, 48, and 72 hpi. Samples were suspended in cell culture media prior to centrifugation and qRT-PCR analysis of supernatant with 6 replicates per matrix type and 4 repeated measures per replicate
[2]DMEM = Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)
[3]SBM = soybean meal
[4]DDGS = Dried distiller's grain with solubles
[5]FEED = complete swine gestation diet
[a,b]Means lacking a common superscript differ ($P < 0.05$)

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