

2021

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Recommended Citation

Blomme, Allison K.; Gebhardt, Jordan T.; Jones, Cassandra K.; Woodworth, Jason C.; Poulsen Porter, Elizabeth; Bai, Jianfa; and Paulk, Chad B. (2021) "Effect of Benzoic Acid and Essential Oil Blends on Viral Load in Swine Feed and Vitamin Premix," *Kansas Agricultural Experiment Station Research Reports*: Vol. 7: Iss. 11. <https://doi.org/10.4148/2378-5977.8212>

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Allison K. Blomme, Jordan T. Gebhardt,¹ Cassandra K. Jones,² Jason C. Woodworth,² Elizabeth G. Poulsen Porter,¹ Jianfa Bai,¹ and Chad B. Paulk

Summary

Feed has been shown to harbor viable virus of interest to swine producers over an extended period of time. The use of mitigants and kill steps have been investigated with variable results. This study investigated 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 a vitamin premix (Exp. 2). 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, in addition to a control with no feed additive to test the mitigant's effect on PEDV, PRRSV, and SVA detection. For Exp. 2, a vitamin premix without chemical treatment acted as the control and the other treatment was the vitamin premix treated with 2.68% EO, with both used to determine PEDV detection. The inoculated feed or premix was stored for up to 15 d with sampling points at 2, 5, and 15 d post-inoculation. Samples were analyzed using a triplex qRT-PCR to detect changes in RNA quantities for all three viruses. A significant treatment \times day interaction was observed in the feed for both PEDV ($P = 0.008$) and SVA ($P < 0.001$). Per the decreased cycle threshold (Ct) value, the 0.5% BA treatment had higher ($P < 0.05$) measurements of detectible PEDV on d 2 and 5, and lower amounts of detectible PEDV on d 15, as compared to the control. The 0.5% BA treated feed had lower ($P < 0.05$) detectable SVA on d 2 but higher detectible SVA on d 15 compared to the control. There was no evidence of difference in detectable PRRSV between treatments. During this experiment, PEDV and SVA showed a degradation over time with rates of degradation varying between treatments. Increasing time from d 2 to 15 decreased (quadratic, $P = 0.038$) detectable PRRSV. The use of the EO in the vitamin premix had no evidence of a treatment \times day interaction, treatment effect, or degradation over time. In conclusion, the use of 0.5% BA had an increased PEDV Ct on d 15 compared to the control (33.8 vs. 32.7 Ct, respectively). However, the use of BA and EO mitigant in this model did not provide consistent evidence for increased viral degradation, but viral load was reduced in the feed matrix over time.

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Introduction

Investigations into the cause of the 2013 porcine epidemic diarrhea virus (PEDV) outbreak in North American swine herds led to feed being identified as a likely vector of disease.³ Studies in subsequent years have shown that other viruses of interest to swine producers are able to survive in feed or feed ingredients over time.⁴ This contaminated feed can cause disease in pigs; therefore, finding ways to reduce viral load would be beneficial in the feed manufacturing process. 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 has been shown to be effective at reducing PEDV in feed, but it does not provide any protection if virus is reintroduced to the feed during a later handling step. Feed additives such as medium chain fatty acids (MCFA), acidifiers, or formaldehyde have also been studied and demonstrated to be beneficial at reducing the amount of detectible virus in feed and feedstuffs.⁵ This study aimed to determine the impact of varying levels of benzoic acid (BA) and an essential oils blend (EO) inclusion in feed and vitamin premix on detectible PEDV, porcine reproductive and respiratory syndrome virus (PRRSV), and Senecavirus A (SVA) using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Materials and Methods

Experiment 1

Treatment structure for the first experiment was arranged as 5×3 factorial with five complete diet-based (FEED) treatments and three timepoints. Treatments consisted of a control with no feed additive, or diet treated with 0.5% benzoic acid (BA, DSM Nutritional Products Inc., Parsippany, NJ), 0.5% BA and 200 ppm essential oil (EO, DSM Nutritional Products Inc., Parsippany, NJ), 0.3% BA and 120 ppm EO, or 0.25% BA and 100 ppm EO. The second factor was day of analysis (d 2, 5, and 15). The diet used was a complete swine gestation diet (Table 1). The diet was analyzed at each time point to confirm it was PEDV, PRRSV, and SVA free as determined by qRT-PCR. All treatments were inoculated with equal amounts of all three viruses as described below. There were three replications per treatment.

Experiment 2

Treatment structure for the second experiment was arranged as a 2×3 factorial with two treatments and three timepoints. Treatments were vitamin premix-based (VIT) and consisted of a control with no additive (but an additional 2.86% limestone) or treatment with 2.86% EO. The second factor was day of analysis (d 2, 5, or 15). The vitamin premix was analyzed by qRT-PCR to confirm it was PEDV-free. Both treat-

³ Pasick, J., Y. Berhane, D. Ojkic, G. Maxie, C. Embury-Hyatt, K. Swekla, K. Handel, J. Fairles, and S. Alexandersen. 2014. Investigation into the Role of Potentially Contaminated Feed as a Source of the First-Detected Outbreaks of Porcine Epidemic Diarrhea in Canada. *Transboundary and Emerging Diseases*. 61:397–410. doi: 10.1111/tbed.12269.

⁴ Dee, S. A., F. v. Bauermann, M. C. Niederwerder, A. Singrey, T. Clement, M. de Lima, C. Long, G. Patterson, M. A. Sheahan, A. M. M. Stoian, V. Petrovan, C. K. Jones, J. de Jong, J. Ji, G. D. Spronk, L. Minion, J. Christopher-Hennings, J. J. Zimmerman, R. R. Rowland, E. Nelson, P. Sundberg, and D. G. Diel. 2018. Survival of viral pathogens in animal feed ingredients under transboundary shipping models. Y. Cao, editor. *PLoS ONE*. 13:e0194509. doi:10.1371/journal.pone.0194509.

⁵ Nichols, G. E.; Gebhardt, J. T.; Jones, C. K.; Woodworth, J. C.; Dritz, S. S.; Bai, J.; Anderson, J. W.; Porter, E.; Sandberg, F. B.; Singrey, A.; and Paulk, C. B. (2020) "Efficacy of Feed Additives Against Swine Viruses in Feed," *Kansas Agricultural Experiment Station Research Reports*: Vol. 6: Iss. 10. doi: 10.4148/2378-5977.8013.

ments were inoculated with PEDV as described below. The guaranteed analysis of the VIT is contained in Table 2. There were three replications per treatment.

Preparation and chemical treatment

Chemical treatments were applied to 100 g of FEED or VIT 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). FEED or VIT was mixed for 15 min with 10 hex nuts to provide adequate agitation. After mixing, three aliquots of 17.5 g from each treatment were placed into three polyethylene bottles per timepoint (250 mL Nalgene, square wide-mouth high-density polyethylene; Thermo Fisher Scientific, Waltham, MA).

Viral isolates and inoculation

All FEED treatments except the negative control were inoculated with 2.5 mL each of PEDV, PRRSV, and SVA. The VIT treatments, except the negative control, were inoculated with 2.5 mL of PEDV. The stock PEDV, PRRSV, and SVA all contained an initial concentration of 10^6 tissue culture infectious dose (TCID)₅₀/mL of their virus.

Inoculation was performed at the Kansas State University College of Veterinary Medicine Virology Laboratory. After the 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 of feed matrix was 10^5 TCID₅₀/g each for PEDV, PRRSV, and SVA.

Real time PCR analysis

Real time PCR was conducted at the Molecular Diagnostic Research and Development section of the Kansas State Veterinary Diagnostic Laboratory. Separate bottles were analyzed on d 0, 2, 5, and 15 post-laboratory inoculation. Samples were stored at room temperature until addition of 100 g phosphate buffered saline (PBS; pH 7.4 1×, Gibco, Thermo Fisher Scientific, Waltham, MA) was added to the bottles of FEED or VIT at appropriate time points. After PBS addition, the samples were swirled to ensure even mixing and stored at 39°F for 24 h at which point supernatant was collected and aliquoted for further analysis. The aliquots were stored at -4°F until qRT-PCR was performed.

After collection of d 15 post-laboratory inoculation aliquots, qRT-PCR on all samples was conducted. Fifty μ L of supernatant from each sample was 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 μ L. Controls of both the FEED and VIT without additives or virus were collected at each time point and included in the PCR to confirm the PEDV, PRRSV, and SVA negative status of the feedstuffs. One negative extraction control consisting of all reagents except the sample was included in each extraction. Positive controls of each stock virus were also included with each extraction. Analyzed values represent cycle threshold (Ct) at which virus was detected. Larger Ct values indicate more cycles must proceed before viral genetic material is detected, thus the original sample has lower quantities of viral genetic material.

Statistical analysis

Data were analyzed using PROC GLIMMIX (SAS Institute, Inc., Cary, NC) to determine the main effects of additive, time, and their interaction on PEDV, PRRS, and SVA Ct values in FEED and PEDV Ct values in VIT 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 being non-detectible for PEDV and PRRSV on d 15 and having a studentized residual value 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 $P \leq 0.05$.

Results and Discussion

Experiment 1

There was a treatment \times day interaction for the FEED matrix ($P = 0.008$) in which the 0.5% BA treatment had increased amounts of detectible PEDV RNA on d 2 and 5 but had decreased amounts on d 15 compared to the control ($P < 0.05$; Table 3). All other treatments had similar detectible amounts of PEDV RNA compared to the control on all days. During this experiment, PEDV RNA degraded over time with rates of degradation varying between treatments.

For detectible PRRSV RNA, there was no evidence for a treatment \times day interaction or a dietary treatment main effect ($P > 0.05$). For PRRSV in FEED, there was decreased (quadratic, $P < 0.038$) detectible RNA as sample storage increased to d 15. The PRRSV Ct increased by 3.2 Ct units from d 2 to d 5 and 3.9 Ct units from d 5 to 15.

There was a treatment \times day interaction for SVA RNA ($P < 0.001$). On d 2, all of the treatments had less detectible SVA RNA than the control. All the BA and EO treatments were similar to the control on d 5. In addition, the 0.5% BA treatment had more detectible SVA RNA than the 0.25% BA with 100 ppm EO treatment. On day 15, the 0.5% BA with 200 ppm EO treatment had similar amounts of detectible SVA RNA to the control, with all of the other treatments having more detectible SVA RNA compared to the control. The 0.5% BA with 200 ppm EO treatment also had less detectible SVA RNA than the 0.5% BA treatment ($P < 0.05$).

Experiment 2

For the vitamin premix matrix, there was no evidence for a treatment \times day interaction on detectible amounts of PEDV RNA ($P = 0.962$; Table 4). Main effects of treatment and day did not affect detectible PEDV RNA in a vitamin premix matrix ($P > 0.05$).

In summary, there was no conclusive evidence of reduction of viral load in feed or vitamin premix with the use of benzoic acid and an essential oils blend with this study. There was a decrease in amount of detectible genetic material for all three viruses from d 2 to d 15 in the feed matrix. This decline led to PRRSV being undetectable in several bottles on d 15 and an increase in Ct for both PEDV and SVA. The SVA had the most detectible RNA in the feed matrix over the study period, and it did not demonstrate the same magnitude of Ct change from d 2 to d 15 as PEDV and PRRSV. This smaller difference in Ct value over time could indicate greater survival of SVA over the same period. The use of EO in the vitamin premix had a higher starting Ct value than PEDV

in the feed, but it did not demonstrate a difference from the control or as large of a decrease over time.

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

Ingredient, %	Swine gestation diet
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
Essential oil ⁵	+/-
Benzoic acid ⁶	+/-

¹Each pound contains 9,979 mg Mn, 33,112 mg Fe, 33,112 mg Zn, 4,990 mg Cu, 198 ppm I, and 198 ppm Se.

²Each pound contains 2,000 IU vitamin E, 450 mg vitamin B₆, 50,000 mg choline, 20 mg biotin, 150 mg folic acid, 40 ppm chromium, and 9,921 ppm L-carnitine.

³Each pound contains 750,000 IU vitamin A, 300,000 IU vitamin D₃, 8,000 IU vitamin E, 6 mg vitamin B₁₂, 600 mg menadione, 1,500 mg riboflavin, 5,000 mg D-pantothenic acid, and 9,000 mg niacin.

⁴Ronozyme HiPhos (GT) 2700 (DSM Nutritional Products Inc., Parsippany, NJ).

⁵Essential oils blend, (DSM Nutritional Products Inc., Parsippany, NJ) added to complete diet at 200 ppm, 120 ppm, or 100 ppm in appropriate treatments.

⁶Benzoic acid (DSM Nutritional Products Inc., Parsippany, NJ) added to complete diet at 0.50%, 0.30%, or 0.25% in appropriate treatments.

Table 2. Guaranteed analysis of vitamin premix (Exp. 2)

Item	Inclusion per lb
Vitamin A	750,000 IU
Vitamin D ₃	300,000 IU
Vitamin E	8,000 IU
Vitamin B ₁₂	6.0 mg
Menadione	600 mg
Riboflavin	1,500 mg
D-pantothenic acid	5,000 mg
Niacin	9,000 mg

Table 3. Interactive effect of treatment and day on viral Ct values (Exp. 1)¹

	Day			SEM	Treatment × day	<i>P</i> ≤		Treatment
	2	5	15			Day		
						Linear	Quadratic	
PEDV				0.38	0.008	0.001	0.001	0.135
Control	28.3 ^e	31.4 ^e	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 ^e	33.5 ^{a,b,2}					
0.25% BA, 100 ppm EO	29.0 ^e	31.1 ^{c,d}	33.0 ^{a,b}					
PRRSV				1.54	0.672	0.001	0.038	0.847
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 ²					
0.25% BA, 100 ppm EO	31.5	34.5	36.2					
SVA				0.18	0.001	0.001	0.131	0.062
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}					

¹ An initial tissue culture (2.5 mL of each diluted virus inoculum, 10⁵ TCID₅₀/mL) was inoculated into 17.5 g of sow gestation diet (FEED) treated with benzoic acid (BA, DSM Nutritional Products Inc., Parsippany, NJ) and/or an essential oil blend (EO, DSM Nutritional Products Inc., Parsippany, NJ), or no chemical treatment with three replications per treatment unless otherwise noted. Data reported as cycle threshold (Ct) required to detect viral genetic material. Higher Ct values indicate less viral genetic material detected.

² One outlier with Ct of 45 and studentized residual > 4 removed from analysis resulting in *n* = 2.

^{a,b,c,d,e,f,g} Means without common superscript within matrix-virus group are significantly different.

PEDV = porcine epidemic diarrhea virus. PRRSV = porcine reproductive and respiratory syndrome virus. SVA = Senecavirus A.

Table 4. Main effect of treatment on PEDV Ct value in a vitamin premix (Exp. 2)¹

	Day			SEM	Linear ²	Quadratic	Treatment		SEM	P ³ =
	2	5	15				Control	2.68% EO		
PEDV	33.7	34.3	34.6	0.48	0.279	0.533	34.8	33.6	0.40	0.066

¹An initial tissue culture (2.5 mL of diluted virus inoculum, 10⁵ TCID₅₀/mL) was inoculated into 22.5 g of vitamin premix (VIT) treated with an essential oil blend (EO, DSM Nutritional Products Inc., Parsippany, NJ), or no chemical treatment with nine replications per treatment unless otherwise noted. Data reported as cycle threshold (Ct) required to detect viral genetic material. Higher Ct values indicate less viral genetic material detected.

²Main effects of day. There was no evidence of a treatment × day interaction ($P = 0.962$).

³Main effects of treatment.