



DR. MEGAN C. NIEDERWERDER (Orcid ID : 0000-0002-6894-1312)

DR. SCOTT DEE (Orcid ID : 0000-0001-8691-3887)

DR. RAYMOND ROWLAND (Orcid ID : 0000-0002-7843-2968)

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Mitigating the risk of African swine fever virus in feed with antiviral chemical additives

Running Title: Use of feed additives to inactivate ASFV in feed

Megan C. Niederwerder^{1*}, Scott Dee², Diego G. Diel³, Ana M.M. Stoian¹, Laura A. Constance¹, Matthew Olcha¹, Vlad Petrovan¹, Gilbert Patterson⁴, Ada G. Cino-Ozuna¹ and Raymond R.R. Rowland¹

1. Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, United States of America

2. Pipestone Applied Research, Pipestone Veterinary Services, Pipestone, Minnesota, United States of America

3. Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States of America

4. Center for Animal Health in Appalachia, Lincoln Memorial University, Harrogate, Tennessee, United States of America

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*Corresponding author: Megan C. Niederwerder

Mailing Address: L-227 Mosier Hall, College of Veterinary Medicine, Kansas State University, 1800 Denison Ave, Manhattan, KS 66506

Email: mniederwerder@vet.k-state.edu; Phone: 785-532-4663

Summary

African swine fever (ASF) is currently considered the most significant global threat to pork production worldwide. Disease caused by the ASF virus (ASFV) results in high case fatality of pigs. Importantly, ASF is a trade-limiting disease with substantial implications on both global pork and agricultural feed commodities. ASFV is transmissible through natural consumption of contaminated swine feed and is broadly stable across a wide range of commonly imported feed ingredients and conditions. The objective of the current study was to investigate the efficacy of medium chain fatty acid and formaldehyde based feed additives in inactivating ASFV. Feed additives were tested in cell culture and in feed ingredients under a transoceanic shipment model. Both chemical additives reduced ASFV infectivity in a dose-dependent manner. This study provides evidence that chemical feed additives may potentially serve as mitigants for reducing the risk of ASFV introduction and transmission through feed.

Keywords: African swine fever virus, ASFV, animal feed, food additives, anti-infective agents, virus inactivation, swine diseases, ships, domestic pig

Introduction

African swine fever (ASF) is a contagious disease of swine resulting in high case fatality associated with significant hemorrhage. The causative agent, ASF virus (ASFV), is an enveloped double-stranded DNA virus and the only member of the genus *Asfivirus* in the family *Asfarviridae* (Galindo & Alonso, 2017). ASFV is a large and complex unique virus with unknown correlates of protection and protective antigens, which has created challenges for infection control and vaccine development (Rock, 2017). Currently, there are no commercially available vaccines and no effective treatments which can be administered to pigs for ameliorating disease. Control of ASF is focused on biosecurity to prevent introduction of the virus and large-scale culling of infected or high-risk animals to contain virus spread.

In August 2018, ASF was reported for the first time in China (Zhou et al., 2018), the world's largest producer of pigs. Subsequently, the virus spread at a rapid rate into 10 new countries in Asia, including Vietnam and South Korea (Kim et al., 2020; Le et al., 2019). Concurrent to the spread in Asia, ASFV also expanded into new areas of Europe, including Slovakia and Belgium (Forth et al., 2019; SHIC, 2019). Recently, the Food and Agriculture Organization (FAO) determined that the current ASF situation is an “unprecedented animal health crisis” and stated that “progressive spread of ASF appears to be inevitable” (FAO, 2020). Although recent experimental evidence has shown promise for potential vaccine candidate efficacy in pigs and wild boar (Barasona et al., 2019; Borca et al., 2020), primary efforts in countries currently negative for the disease are focused on prevention of virus entry at the borders and on swine farms.

Shortly after the 2013 introduction of porcine epidemic diarrhea virus (PEDV) in the U.S., the global trade of feed ingredients was recognized as a potential risk factor for the introduction and transboundary spread of porcine viral diseases (Niederwerder & Hesse, 2018). Over the last several years, importation of select feed ingredients has increased from China to the U.S. through the San Francisco Port of Entry, with over twice the volume imported in 2018 (approximately 31,842 metric tons) compared to 2013, when approximately 13,026 metric tons were imported (Stoian et al., 2020). Experimentally, ASFV has demonstrated broad stability in a wide-range of feed ingredients in a transoceanic shipment model, which replicates real-life temperature and humidity conditions. Specifically, the virus maintained infectivity throughout the 30-day simulated voyage in 75% of the

feed or feed ingredients tested with a half-life of approximately 12.2 days (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian, Petrovan, Jones, De Jong, et al., 2018; Stoian et al., 2019). Furthermore, ASFV is transmissible through feed, following the natural consumption of contaminated plant-based ingredients, with increased probability of infection being demonstrated after repeated exposures over time (Niederwerder et al., 2019).

Combining experimental evidence with field reports of contaminated feed contributing to ASFV spread in affected countries (Olsevskis et al., 2016; Wen et al., 2019), mitigating the risk of feed as a possible route for ASFV entry is a priority for negative countries and regions. Mitigation of bacterial and viral pathogens in poultry, cattle and swine feed through the use of chemical feed additives has been previously reported for *Salmonella enterica*, PEDV, avian influenza virus, *Escherichia coli*, and porcine deltacoronavirus (Amado, Vazquez, Fucinos, Mendez, & Pastrana, 2013; Cottingim et al., 2017; Toro, van Santen, & Breedlove, 2016; Trudeau et al., 2016). For example, both medium chain fatty acid and formaldehyde based feed additives have demonstrated efficacy in reducing PEDV in contaminated feed and feed manufacturing equipment (S. Dee et al., 2016; Gebhardt et al., 2018). Viral inactivation by formaldehyde is associated with protein and nucleic acid cross-linking (Sabbaghi, Miri, Keshavarz, Zargar, & Ghaemi, 2019) whereas viral inactivation by medium chain fatty acids are associated with disruption of the viral envelope integrity (Thormar, Isaacs, Brown, Barshatzky, & Pessolano, 1987).

The objective of the current study was to investigate two liquid feed additives, including a medium chain fatty acid based additive and a formaldehyde based additive, for efficacy against ASFV in a cell culture model and in a feed ingredient shipment model. In general, both chemical additives demonstrated evidence of reducing ASFV infectivity, with data suggesting dose-dependent efficacy.

Materials and Methods

Cells, viruses and chemical additives

ASFV BA71V was propagated and titered on Vero cells whereas ASFV Georgia 2007 was derived from splenic homogenate and titered on porcine alveolar macrophages (PAMs). Additives included a commercially available feed additive composed of 37% aqueous formaldehyde and propionic acid (Sal CURB[®], Kemin Industries, Inc., Des Moines, Iowa, USA) and a blend of three commercially available medium chain fatty acids (MCFA, Sigma-Aldrich, St. Louis, Missouri, USA).

The MCFA blend included an equal volume ratio (1:1:1) of hexanoic acid (C6), octanoic acid (C8) and decanoic acid (C10).

For testing in cell culture, dilutions of the formaldehyde based additive were prepared in Minimum Essential Medium (Corning® Eagle's MEM; Fisher Scientific) supplemented with fetal bovine serum (FBS), antibiotics and antimycotics. For the MCFA based additive, an initial 20% MCFA stock solution was prepared in dimethyl sulfoxide (DMSO; Fisher BioReagents, Pittsburgh, Pennsylvania, USA) to prevent precipitation. Subsequent dilutions of the MCFA/DMSO stock were prepared in MEM supplemented with FBS, antibiotics and antimycotics. Prior to testing for antiviral activity, each chemical additive was tested at several dilutions (2.0%, 1.0%, 0.5%, 0.25%, 0.13%, 0.06%) on non-infected Vero cells to confirm the lack of chemical-induced cytotoxicity in cell culture.

***In vitro* ASFV BA71V testing**

Dilutions of each chemical additive between 0.03% and 2.0% were mixed with an equal volume of ASFV BA71V (titer 10^6 50% tissue culture infectious dose per ml, TCID₅₀/ml). Serial 10-fold dilutions of each chemical/virus combination were prepared in triplicate for titration on confluent monolayers of Vero cells. Positive controls included BA71V mixed with an equal volume of media. Samples treated with the formaldehyde based additive were incubated for 30 min at room temperature prior to titration based on previous inactivation experiments using vaccinia virus (data not shown). ASFV titers were determined by immunofluorescence assay (IFA) on Vero cells. Briefly, after 3 days of incubation at 37°C, Vero cells were washed three times with phosphate-buffered saline (PBS) and fixed with 80% acetone. Monoclonal antibody directed at ASFV p30 (Petrovan et al., 2019) was added at a dilution of 1:6000 (ascites fluid). After 1 hr incubation at 37°C, the plate was washed 3 times with PBS and goat anti-mouse antibody (AlexaFluor 488, Life Technologies) was added at a 1:400 dilution and fluorescence observed under the inverted microscope. The TCID₅₀/ml was calculated according to the method of Reed and Muench (Reed & Muench, 1938).

Feed shipment model

Nine animal feed ingredients or complete feed known to support survival of ASFV Georgia 2007 for at least 30 days of transoceanic shipment conditions were selected for the current study (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian,

Petrovan, Jones, De Jong, et al., 2018; Stoian et al., 2019). Feed or ingredients included conventional soybean meal, organic soybean meal, soy oil cake, choline, moist dog food, moist cat food, dry dog food, pork sausage casings and complete feed in meal form. Table 1 shows the quantity of these ingredients imported into the U.S. from Europe over the last seven years, with substantial increases in the volume of these commodities imported in 2018 and 2019 compared to previous years. Feed and feed ingredients were gamma-irradiated (minimum absorbed dose of 25 kilograys) prior to use. Five grams of each ingredient were added to 50 ml conical tubes and organized into 6 different treatment groups (Figure 1).

At 0 days post-contamination (dpc), samples in groups A and B were treated with the corresponding chemical additive (50 μ l MCFA/sample or 16.5 μ l formaldehyde/sample) and the tubes were vortexed for 10 sec. Inclusion rates of 1% MCFA based additive and 0.33% formaldehyde based additive were selected due to previous work with biosafety level 2 viruses in feed shipment models (data not shown). All samples from all groups were then inoculated with 100 μ l of ASFV Georgia 2007 (corresponding to a final concentration of 10^5 TCID₅₀/sample) and vortexed for 10 sec. Solid caps were replaced with vented caps to facilitate temperature and humidity exchange. Samples were placed in an environmental chamber (Model 3911, Thermo Scientific Forma, Waltham, MA) programmed to simulate transoceanic shipment conditions as previously described (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian, Petrovan, Jones, De Jong, et al., 2018; Stoian et al., 2019). Briefly, temperature and humidity values fluctuated every 6 hr based on historical meteorological data from April 5, 2011 to May 4, 2011 to model a 30-day shipment from Warsaw, Poland to Des Moines, IA, USA (Figure 2).

On 1, 8, and 17 dpc, duplicate samples from group A, group B, positive control, and negative control were removed from the environmental chamber and processed for testing. At 28 dpc, samples in groups C and D were treated with the corresponding chemical additive. At 30 dpc, all remaining samples were removed and processed for testing. For processing, 15 ml of sterile PBS with antibiotics and antimycotics were added to each tube. Vented caps were replaced with solid caps and the samples vortexed for 10 sec, followed by centrifugation at 10,000g for 5 min at 4°C. Supernatant from each sample was stored at -80°C.

ASFV PCR

For detection of ASFV by qPCR, nucleic acid was extracted using the MagMAX™ Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, MA). Negative and positive extraction controls were included on each plate. Briefly, 50 µl of feed ingredient supernatant was combined with 20 µl Bead mix (containing Lysis/Binding Solution, Carrier RNA, and 100% isopropanol) on a U-bottom 96-well plate. The plate was mixed for 1 min on an orbital shaker prior to cell lysis using 130 µl Lysis/Binding solution followed by another 5 min of mixing. Beads were captured on a magnetic stand and washed twice using 150 µl Wash Solution 1 and 2. The final elution volume was 50 µl. Extracted test samples and controls were used immediately for the PCR assay using primers and probe designed to amplify a conserved region of ASFV p72 (King et al., 2003) as previously described in detail (Niederwerder et al., 2019). For each plate, a standard curve was generated with 10-fold serial dilutions of a 10⁶ TCID₅₀/ml ASFV Georgia 2007 stock. Data analysis was performed using CFX96 software and results were reported as the cycle threshold (Ct) values per 20 µl PCR reaction.

ASF Georgia 2007 virus isolation

For detection of infectious ASFV, PAMs were collected from 3-5 week old pigs by lung lavage. PAMs were cultured for two days in RPMI media (Gibco, ThermoFisher Scientific) supplemented with 10% FBS and antibiotics at 37°C in a 5% CO₂ incubator. Each feed ingredient supernatant was 2-fold serially diluted in RPMI media in triplicate prior to being added to washed monolayers of PAMs in 96-well plates and incubated for 1 hr at 37°C. Plates were washed and RPMI media replaced prior to a 4 day incubation at 37 °C. Following incubation, cells were fixed and IFA was performed as described above. The log₁₀ TCID₅₀/ml was calculated according to the method of Spearman and Karber (Finney, 1964).

ASFV Georgia 2007 bioassay

All samples collected on 30 dpc with detectable ASFV DNA on qPCR but negative for infectious virus on PAMs were tested in a pig bioassay. A total of 24 weaned barrows (average age 24.0 ± 0.4 days) were obtained from a high-health commercial source. All pigs were housed in individual 1.9 m² pens in a 66 m² large animal room at the Biosecurity Research Institute under biosafety level 3 agriculture (BSL-3Ag) containment conditions. Each stainless-steel pen was raised, contained slotted fiberglass flooring, and was separated by at least 1.5 m from other pens within the

room. Three sides of the pen were solid with the fourth side consisting of bars and a gate. The room was environmentally controlled and complete exchange of air occurred 14.5 times/hr. Six pigs were housed within the room at any given time with one pig being maintained as a negative control to confirm the lack of cross-contamination and aerosol transmission between pens. To prepare the inoculum, supernatant from duplicate feed samples collected at 30 dpc were centrifuged and pooled to create a 1-ml inoculum for intramuscular injection. Each pig received one or two 1-ml injections in the hind limbs for testing up to two different feed sample types. This experimental design was intended to minimize the number of pigs used in bioassays.

After 3-4 days of acclimation upon arrival to the BSL-3Ag animal facility, all pigs were inoculated or mock-inoculated (supernatant from negative control complete feed samples) with the 1-ml suspensions as described above. Pigs were monitored daily by a veterinarian or veterinary assistant for clinical signs of ASF, including fever, lethargy or depression, dyspnea or tachypnea, diarrhea, weight loss or muscle wasting, hyperemia or hemorrhage, difficult ambulation or ataxia. At 6 days post-inoculation (dpi), all pigs were humanely euthanized by intravenous pentobarbital injection and tissues were collected for diagnostic testing. Specifically, serum and splenic homogenate were tested for the presence of ASFV DNA on qPCR and splenic homogenate was tested for viable ASFV on virus isolation.

Splenic lysate for diagnostic testing of pigs was created by mincing spleen and passing it through a cell strainer after adding PBS with antibiotics and antimycotics. Suspensions were centrifuged at 4,000g for 30 min prior to transferring the supernatant into a 50 ml conical tube for storage at 4°C. Cell pellets were resuspended in sterile PBS with antibiotics and antimycotics followed by 3 freeze-thaw cycles. Cell suspensions were centrifuged again at 4,000g for 30 min and supernatants from each pig were pooled for testing. Diagnostic testing of splenic lysate and serum by quantitative PCR was performed as described above. For virus isolation of splenic lysate on PAMs, 2-fold serial dilutions were prepared in RPMI media and four dilutions (1:15, 1:30, 1:60, 1:120) tested as described above.

Results

Cell-culture efficacy of chemical feed additives

The results for each chemical additive are shown in Figure 3. Overall, there was a dose dependent reduction in virus titer post-exposure to each chemical additive, with higher inclusion levels of MCFA required to decrease virus titers below the level of detection on IFA.

For the formaldehyde additive, an inclusion rate as low as 0.03% resulted in an 82.2% reduction in virus concentration; 5.4 log₁₀ TCID₅₀/ml after chemical exposure compared to 6.2 log₁₀ TCID₅₀/ml for the positive control. At 0.3% inclusion, the ASFV titer was reduced by 3.5 log₁₀ TCID₅₀/ml with greater than 99.9% reduction in virus concentration compared to the untreated positive control. Increasing the percent inclusion to 0.35% reduced the virus concentration to below the limit of detection in cell culture by IFA (Fig 3A).

For the MCFA additive, an inclusion rate as low as 0.13% resulted in an 82.2% reduction in virus concentration; 5.4 log₁₀ TCID₅₀/ml after chemical exposure compared to 6.2 log₁₀ TCID₅₀/ml in the positive control. At 0.6% inclusion, viral titers were reduced by 3.8 log₁₀ TCID₅₀/ml with greater than 99.9% reduction in virus concentration compared to the untreated positive control. Inclusion rates at and above 0.7% reduced viral titers to below the level of detection on Vero cells (Fig 3B).

Feed shipment model efficacy of chemical additives

Environmental conditions throughout the transoceanic shipment model (Figure 2) were consistent with previous reports (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian, Petrovan, Jones, Jong, et al., 2018; Stoian et al., 2019). Overall, feed ingredients were exposed to moderate humidity (mean ± SD, 74.1 ± 19.2%) and moderate temperature (mean ± SD, 12.3 ± 4.7°C) climatic conditions.

All duplicate feed samples collected on 1, 8, 17 and 30 were tested by qPCR to quantify ASFV nucleic acid stability over time and degradation associated with exposure to feed additives. Mean 40-Ct values of duplicate samples are shown in Figure 4. All ASFV-inoculated feed samples had detectable nucleic acid (Ct < 40) at each time point tested, including all those samples exposed to feed additives. All negative control samples lacked detectable ASFV nucleic acid on 1, 8, 17 and 30 dpc (Figure 4I; Ct ≥ 40).

Over the 30-day time course of the shipment model, ASFV nucleic acid was generally stable across the 9 untreated ingredients. Exposure to MCFA (groups A and C) did not reduce the quantity of detectable ASFV nucleic acid in feed ingredients, with similar Ct values to the positive controls.

However, several ingredients had notable reductions of ASFV nucleic acid after exposure to formaldehyde (groups B and D), including conventional soybean meal, organic soybean meal, soy oilcake, dry dog food, moist cat food, and moist dog food starting as early as 1 dpc (Figure 4A-F). For example, on 1 dpc, the mean 40-Ct value in dry dog food treated with formaldehyde at 0 dpc was 4.9 compared to 13.4 in the positive control. The effect of formaldehyde exposure on ASFV genome detection was less notable in choline, pork sausage casings and complete feed (Figure 4G-I). When formaldehyde treatment occurred at 28 dpc (group D), the effect on nucleic acid was similar to group B in soy products but had less effect on ASFV DNA in pet foods.

All feed ingredient samples collected on 30 dpc were titrated in triplicate on PAMs for quantification of infectious ASFV (Table 2). Positive control samples for all 9 feed ingredients had ASFV titers similar to our previously published work (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian, Petrovan, Jones, De Jong, et al., 2018) and ranged between $10^{2.7} - 10^{3.2}$ TCID₅₀. All duplicate feed ingredients treated with MCFA or formaldehyde (groups A-D) had no infectious ASFV detected at 30 dpc. Additionally, negative control complete feed samples were negative for ASFV on IFA.

All MCFA or formaldehyde treated samples had detectable ASFV DNA on PCR but lacked detectable ASFV on virus isolation at 30 dpc. Thus, all treated samples were tested in a pig bioassay. Each pig received either 1 or 2 samples for testing. Pigs were tested in groups of six, with one pig receiving the complete feed negative control. No overt clinical signs of ASF were noted during the monitoring period and at 6 dpi, all pigs were euthanized and tested for ASFV infection using multiple diagnostic assays. Out of the 24 pigs utilized for bioassays, a single pig had evidence of ASFV infection. The positive pig had received two samples, including organic soybean meal and dry dog food treated with MCFA at 28 dpc from group C. It is unknown if one or both samples maintained infectious ASFV at 30 dpc. All remaining pigs lacked evidence of ASFV infection on serum PCR, spleen PCR and spleen VI.

Discussion

African swine fever is currently considered the greatest global threat to pork production with significant efforts being focused on preventing entry into new herds and countries. As the worldwide trade in feed ingredients has recently been recognized as a route for transboundary disease spread,

tools for mitigating the risk of ASFV in feed are needed. In the current study, we tested two feed additives with different active ingredients and modes of action for efficacy against ASFV in commonly imported commodities (Table 1). Overall, inclusion of a MCFA or formaldehyde based additive in contaminated feed ingredients reduced ASFV infectivity.

For both the formaldehyde and MCFA based additives, there were evidence of dose-dependent efficacy *in vitro*. Inclusion rates of 0.35% and 0.7% were necessary to reduce viral titers below the level of detection for formaldehyde and MCFA based additives, respectively. Considering the Environmental Protection Agency virucide requirements of ≥ 4 log reduction in virus titers (EPA, 1981), it is interesting to note that 0.3% formaldehyde based and 0.6% MCFA based additive inclusion resulted in reductions of virus concentration by 3.5 and 3.8 \log_{10} TCID₅₀/ml, respectively. This formaldehyde inclusion is similar to the current FDA-approved formaldehyde rate for maintaining animal feeds or ingredients as *Salmonella* negative for 21 days (FDA, 2019). In general, approximately twice the volume of the MCFA based additive was required to obtain inactivation results similar to the formaldehyde based additive. The *in vitro* cell culture data suggest that inclusion rates lower than what was tested in the feed shipment model (0.33% formaldehyde based and 1.0% MCFA based) may be effective. However, testing of different inclusion rates was only performed on the cell-culture adapted ASFV strain BA71V and additional dose response investigations are warranted for ASFV Georgia 2007 to identify the lowest effective inclusion rate for each chemical feed additive.

A noteworthy finding in this study is the presence of detectable ASFV DNA by qPCR in all samples treated with MCFA or formaldehyde, despite those samples being primarily negative for infectious virus on virus isolation and pig bioassay. This is important as inactivation criteria for feed additive efficacy against ASFV should not be reliant on a lack of DNA detection on PCR. Due to nucleic acid stability and detection throughout the 30 days in all samples, qPCR would be an appropriate tool for diagnostic screening of feed samples at high risk for ASFV contamination, with confirmatory testing of positive samples on virus isolation. In the treated samples lacking detectable ASFV on PAMs in this study, the vast majority (34/36) subsequently tested negative for infectious ASFV in pig bioassays. In this model, results on PAMs had similar sensitivity to pig bioassay.

Interestingly, while neither feed additive eliminated ASFV DNA, formaldehyde treatment resulted in consistent reductions of nucleic acid whereas no substantial effect was seen after MCFA treatment. A similar trend was seen with PEDV RNA in feed ingredients treated with 0.33% Sal CURB[®] and 2.0% MCFA (S. Dee et al., 2016), where formaldehyde but not MCFA treated ingredients had significant reductions in PEDV RNA 37 days after treatment. Formaldehyde interacts with nucleic acid through multiple pathways, including DNA denaturation by bond instability and breakage (Srinivasan, Sedmak, & Jewell, 2002), which likely contributes to reduced nucleic acid detection post-exposure. Although the formaldehyde-based feed additive demonstrated inactivation at lower inclusion rates and increased efficacy compared to the MCFA-based additive, it is important to consider the effects of each chemical on pig production, including effects on weight gain and gut microbiome composition (Gebhardt et al., 2020; Greiner et al., 2017; Williams et al., 2018; Zhang, Baek, & Kim, 2019). For example, Williams et al., 2018 reported that formaldehyde treatment of diets for growing pigs was associated with increased relative abundance of *Clostridiaceae* in the fecal microbiome and reduced average daily gain (Williams et al., 2018). When considering the incorporation of feed additives as a strategy for feed biosecurity of individual production systems, potential negative effects and associated costs should be weighed against the benefits of pathogen risk mitigation.

Overall, this study provides the first evidence of feed additives being effective at reducing ASFV infectivity in feed ingredients and provides foundational knowledge for mitigation tools that may be utilized to reduce the risk of ASFV in feed. Further research is warranted to provide additional recommendations on dose and duration of exposure for MCFA and formaldehyde based additives in ASFV-contaminated feed.

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Ethics statement

The authors confirm that the ethical policies of the journal have been adhered to and the appropriate ethical review committee approval has been received. All use of viruses and animals were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use Committee and the Institutional Biosafety Committee.

Conflict of interest statement

The authors have patents pending related to this research.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Volume (metric tons) of selected products and commodities imported into United States ports* from European countries† between 2013 and 2019

Imported Product	HTS Code	2013	2014	2015	2016	2017	2018	2019
DOG AND CAT FOOD, PUT UP FOR RETAIL SALE	2309.10.0010, 2309.10.0090	2,438.2	2,823.7	5,408.9	9,603.5	11,277.4	17,197.2	24,049.0
SOYBEAN OILCAKE AND OTHER SOLID RESIDUES RESULTING FROM THE EXTRACTION OF SOY BEAN OIL, WHETHER OR NOT GROUND OR IN THE FORM OF PELLETS	2304.00.0000	5.0	134.3	1,643.3	108.7	1,720.7	33,166.2	21,973.0
CHOLINE AND ITS SALTS	2923.10.0000	9,700.2	6,377.8	2,740.9	2,777.8	5,401.0	5,468.3	16,375.7
HOG GUTS, BLADDERS & STOMACHS, WHOLE & PIECES THEREOF, FRESH, CHILLED, FROZEN, SALTED, IN BRINE, DRIED OR SMOKED, PREPARED FOR USE AS SAUSAGE CASINGS	0504.00.0020	2,827.3	1,231.2	1,122.1	1,018.2	1,025.9	2,654.0	6,403.2
FLOURS AND MEALS OF SOYBEANS	1208.10.0000, 1208.10.0090	100.1	242.7	476.4	519.5	451.7	947.7	643.9

Total	15,070.9	10,809.6	11,391.5	14,027.8	19,876.8	59,433.4	69,444.7
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Data gathered from the International Trade Commission Harmonized Tariff Schedule (HTS) found at <https://dataweb.usitc.gov/>. Table has been adapted from previous work (Dee et al., 2018; Stoian et al., 2020) and updated by Patterson on March 22, 2020.

*United States ports include Baltimore, MD; Boston, MA; Buffalo, NY; Charleston, SC; Charlotte, NC; Chicago, IL; Cleveland, OH; Columbia-Snake, OR; Dallas-Fort Worth, TX; Detroit, MI; Great Falls, MT; Houston-Galveston, TX; Los Angeles, CA; Miami, FL; Minneapolis, MN; Mobile, AL; New Orleans, LA; New York, NY; Nogales, AZ; Norfolk, VA; Ogdensburg, NY; Philadelphia, PA; Portland, ME; San Diego, CA; San Francisco, CA; San Juan, PR; Savannah, GA; Seattle, WA; St. Albans, VT; St. Louis, MO; Tampa, FL; Washington, DC

†European countries include Austria, Belarus, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, France, Germany, Hungary, Iceland, Ireland, Italy, Liechtenstein, Malta, Netherlands, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, United Kingdom

Tables

Table 2. Detection of ASFV Georgia 2007 by virus isolation and pig bioassay in feed ingredients at the conclusion of the 30-day transoceanic shipment model

Feed Ingredient	No Treatment*	Treatment Groups†			
		A	B	C	D
Conventional soybean meal	10 ^{3.0}	-	-	-	-
Organic soybean meal	10 ^{3.0}	-	-	+‡	-
Soy oilcake	10 ^{3.1}	-	-	-	-
Dry dog food	10 ^{2.7}	-	-	+‡	-
Moist cat food	10 ^{3.0}	-	-	-	-
Moist dog food	10 ^{2.8}	-	-	-	-
Choline	10 ^{3.2}	-	-	-	-
Pork sausage casings	10 ^{2.9}	-	-	-	-
Complete feed	10 ^{2.7}	-	-	-	-
Complete feed (Negative Control)	-	ND	ND	ND	ND

*Data is shown as mean TCID₅₀ calculated from untreated samples collected on 30 dpc.

Each TCID₅₀ calculation was performed from triplicate dilutions on porcine alveolar macrophages. Initial virus inoculation was 10⁵ TCID₅₀.

†All treated samples collected at 30 dpc were negative on virus isolation and were tested in a pig bioassay by intramuscular injection. Bioassay results are shown as positive (+) or negative (-). Four rounds of 6 pigs/round were utilized for bioassays ($n = 24$ pigs), with one pig in each round serving as the negative control. The remaining 5 pigs/round were inoculated with either 1 or 2 feed samples. Samples were combined as follows for each group, including Groups A and B: pork sausage casings, moist and dry dog food, conventional soybean meal and moist cat food, organic soybean meal and complete feed, soy oilcake and choline; Group C: moist dog food, conventional soybean meal and pork sausage casings, organic soybean meal and dry dog food‡, soy oilcake and moist cat food, choline and complete feed; Group D: moist dog food, moist cat food and pork

sausage casings, dry dog food and complete feed, conventional and organic soybean meal, soy oilcake and choline. All pigs were humanely euthanized 6 dpi and tested for ASFV infection by PCR of serum and spleen, and virus isolation of spleen. Samples were considered positive for the presence of infectious ASFV if ≥ 1 diagnostic test was positive. ND, not determined.

‡One pig inoculated with both organic soybean meal and dry dog food from Group C had evidence of ASFV infection. It is unknown if one or both samples had infectious ASFV.

Figure legends

Figure 1. Experimental design to investigate the effects of MCFA or formaldehyde inclusion on ASFV Georgia 2007 in a transoceanic model of shipped feed. Panels show 6 groups designed to determine feed additive efficacy when feed ingredients are treated in the simulated country of origin prior to shipment (Groups A and B, treated on 0 dpc) and upon simulated arrival to the United States post-transport (Groups C and D, treated on 28 dpc). Positive and negative controls were included for each sampling day. A total of 260 feed or ingredient samples were tested in this study (130 samples tested in duplicate), including 20 treated samples/ingredient, 8 positive control samples/ingredient and 8 negative control complete feed samples. Group A included feed and ingredient samples treated with 1% MCFA blend at 0 dpc. Group B included feed and ingredient samples treated with 0.33% formaldehyde based additive at 0 dpc. Group C included feed and ingredient samples treated with 1% MCFA blend at 28 dpc. Group D included feed and ingredient samples treated with 0.33% formaldehyde based additive at 28 dpc. Samples in group A, group B, positive control and negative control were organized into 4 identical batches for testing at 1, 8, 17, and 30 dpc. Samples in groups C and D were tested at 30 dpc.

Figure 2. Environmental conditions of the 30-day transoceanic shipment model. Figure adapted from previous publications (S. A. Dee, Bauermann, Niederwerder, Singrey, Clement, de Lima, Long, Patterson, Sheahan, Stoian, Petrovan, Jones, De Jong, et al., 2018; Stoian et al., 2019) to show temperature (°C, white circles) and relative humidity (% , black circles) values which fluctuated every 6 hours throughout the 30-day period. Land transport periods (Warsaw, Poland to Le Havre, France and New York City, USA to Des Moines, USA) shown in brown and oceanic transport period (Le Havre, France to New York City, USA) shown in blue.

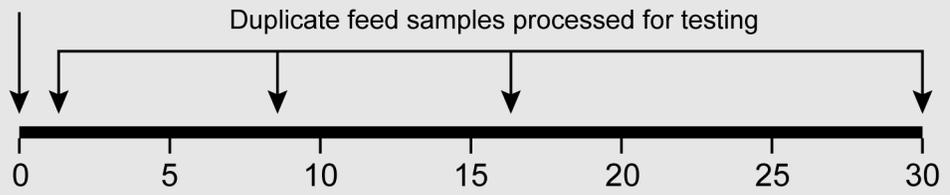
Figure 3. Dose response inactivation curves of ASFV BA71V after exposure to liquid feed additives in cell culture. Data is shown as the \log_{10} TCID₅₀/ml ASFV titer after exposure to different inclusion rates of formaldehyde (A) and MCFA (B) based additives. TCID₅₀/ml calculations performed from triplicate samples. Positive controls are represented by the 0% feed additive inclusion rate. Formaldehyde exposure occurred for 30 min at room temperature prior to virus plating on cells. Virus titers below the limit of detection on IFA are shown as 0 \log_{10} TCID₅₀/ml. Inclusion rates of the formaldehyde based additive tested at 0.35% and higher (0.40, 0.45, 0.50, 1.00, 2.00%) or the MCFA

based additive tested at 0.70 and higher (0.80, 0.90, 1.00, 2.00%) resulted in no detectable virus.

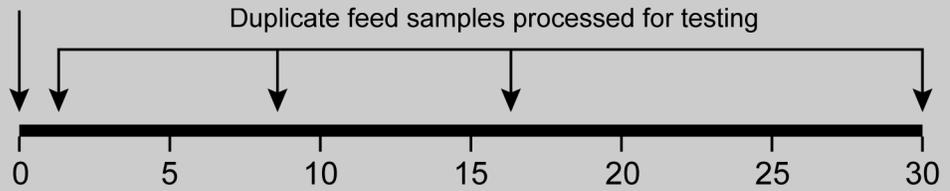
*Results based on 3 separate titration experiments with identical quantities calculated. †Results based on 2 separate titration experiments with mean quantity calculated and shown. ‡Results based on 2 separate titration experiments with identical quantities calculated.

Figure 4. Detection of ASFV Georgia 2007 nucleic acid in feed ingredients over the course of the 30-day transoceanic shipment model. Panels represent conventional soybean meal (A), organic soybean meal (B), soy oilcake (C), dry dog food (D), moist cat food (E), moist dog food (F), choline (G), pork sausage casings (H), and complete feed (I). Data is shown as 40 minus the mean cycle threshold (Ct) values for duplicate samples collected at 1, 8, 17 and 30 dpc. A Ct value ≥ 40 was considered negative. Data is shown for feed ingredients treated with 1% MCFA blend at 0 dpc (Group A; grey squares/grey line), feed ingredients treated with 0.33% formaldehyde based additive at 0 dpc (Group B; black squares/black line), feed ingredients treated with 1% MCFA blend at 28 dpc (Group C; grey circles), feed ingredients treated with 0.33% formaldehyde based additive at 28 dpc (Group D; black circles), feed ingredients without treatment (positive controls; white boxes/dotted line), and complete feed without ASFV-inoculation (negative controls; white circles/black line).

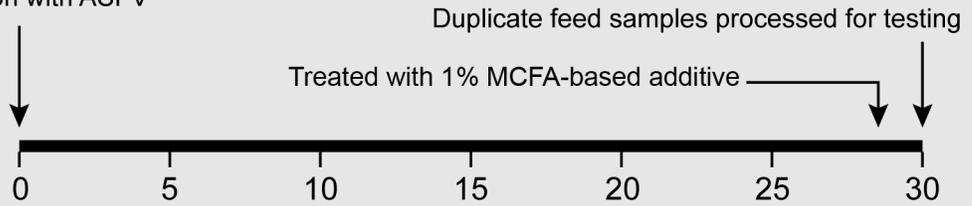
Group A Treated with 1% MCFA-based additive prior to inoculation with ASFV



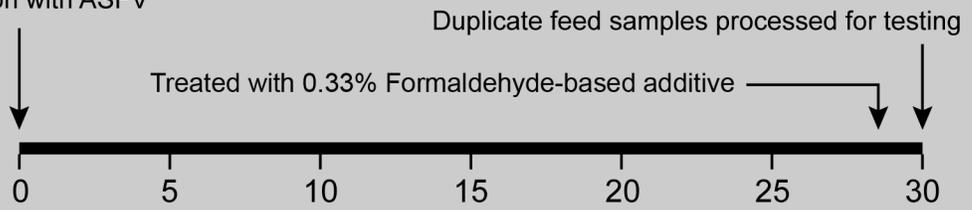
Group B Treated with 0.33% Formaldehyde-based additive prior to inoculation with ASFV



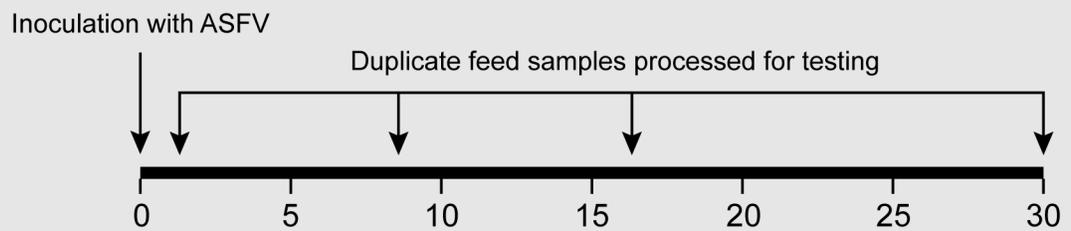
Group C Inoculation with ASFV



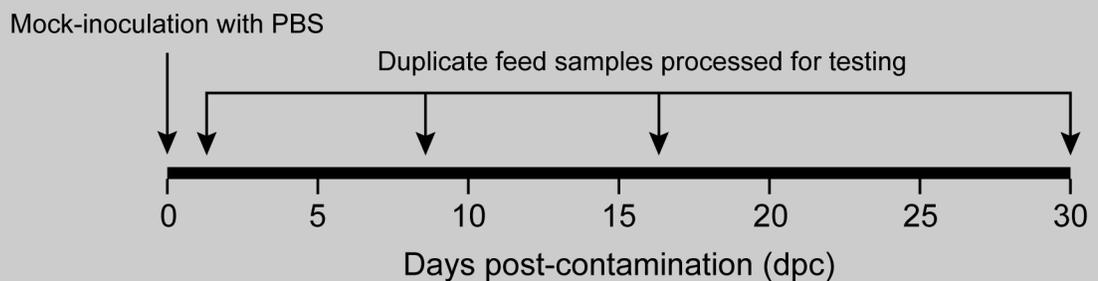
Group D Inoculation with ASFV

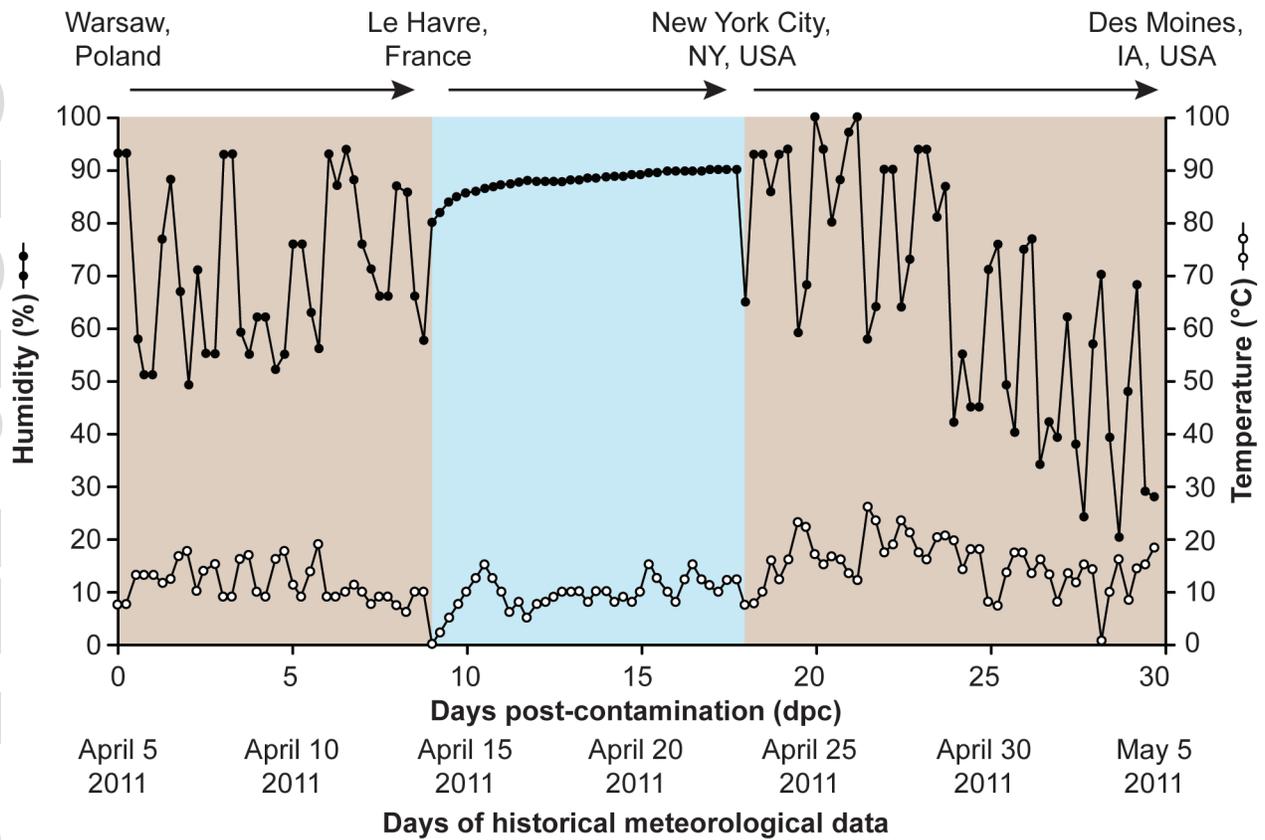


Positive Control

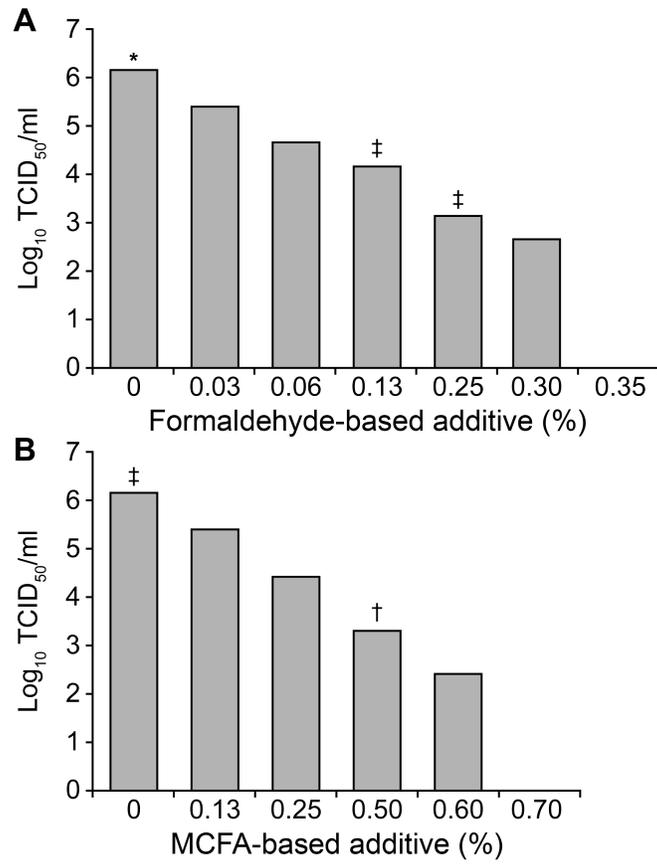


Negative Control

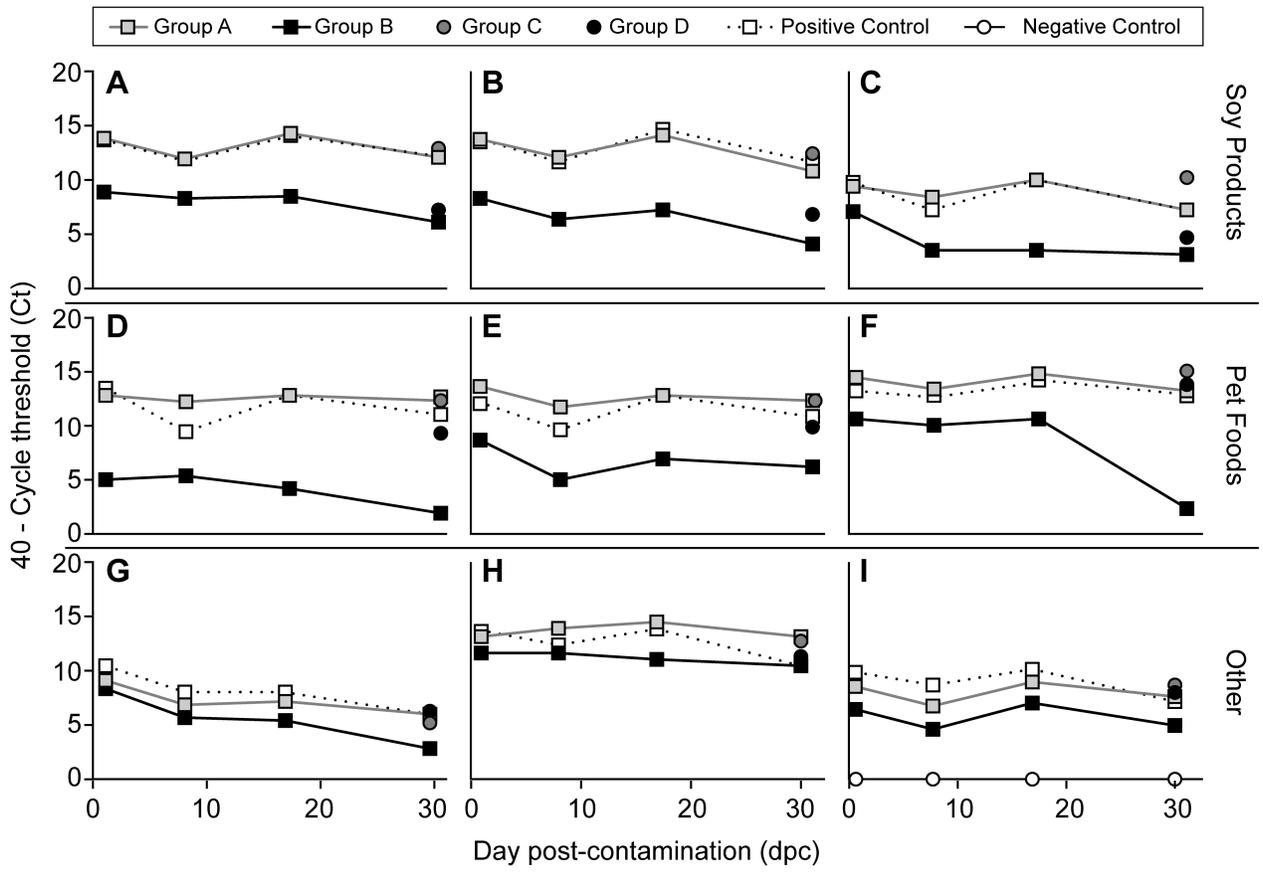




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