Interventional strategies to reduce biological hazards in animal feed

by

Roger Andrew Cochrane

B.A., Franklin College, 2014
M.S., Kansas State University, 2015

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Porcine epidemic diarrhea virus (PEDV) is a heat-sensitive virus that devastated the United States swine industry. Because of its heat sensitivity, it was hypothesized that a pellet mill mimicking commercial thermal processing may mitigate PEDV infectivity. From the results, it was determined that a conditioning time of 30 sec or greater and temperatures above 54.4°C were effective point-in-time kill steps to inactive PEDV in a research setting. However, this does not prevent subsequent recontamination after pelleting as it is a point-in-time mitigation step. To further explore this, various mitigation additives were evaluated to prevent or mitigate PEDV post-pellet contamination in swine feed and ingredients. Various additives were examined across 3 experiments and included mitigation additives of medium chain fatty acids (MCFA), organic acids (OA), essential oils (OA), formaldehyde based products, and sodium bisulfate. From Exp. 1, formaldehyde, medium chain fatty acids (MCFA), essential oils (EO), and organic acid (OA) each decreased detectable PEDV RNA compared to the control ($P<0.05$). Additionally, PEDV stability over time was influenced by matrix as the meat and bone meal and spray-dried animal plasma resulted in a greater ($P<0.05$) quantity of detectable PEDV RNA over 42 days compared to that of the swine diet and blood meal. In Exp. 2, the 1% MCFA inclusion was equally effective at mitigating PEDV as a commercially available formaldehyde product in the complete swine diet. To further explore the effects of MCFA against PEDV, Exp. 3 was conducted to evaluate lower inclusion levels of MCFA and fat sources containing MCFA. It was noted that formaldehyde, 1% MCFA (1:1:1: of caproic, caprylic, and capric acids), 0.66% caproic, 0.66% caprylic, and 0.66% capric acids enhance the RNA degradation of PEDV in swine feed as determined by a bioassay. The MCFA were also evaluated against *Salmonella* Typhimurium, Generic *Escherichia* coli, *Enterotoxigenic Escherichia* coli, and *Campylobacte*r coli. It was

noted that the efficacy of the MCFA varied between each bacteria species with caproic and caprylic being the most effective. Commercial developmental products were also tested and determined that Product A and B provided the lowest MIC values across *Salmonella* Typhimurium, Generic *Escherichia* coli, and Enterotoxigenic *Escherichia* coli ($P < 0.05$). Product A and B were further tested in an animal disease trial utilizing a strain of enterotoxigenic *Escherichia. coli* O149:K91: K88. From d 7 to 14, chlortetracycline, 1:1:1 blend, and Product B, all improved G:F compared to the control ($P<0.05$). This also led to chlortetracycline and Product B having an improvement ($P<0.05$) over the control diet from d 0 to 14. A treatment × day interaction for the enterotoxigenic *E. coli* plate scores was observed ($P < 0.05$), which occurred because of the decrease ($P<0.05$) in plate scores for Product B from d 1 to d 14 and an increase ($P<0.05$) in chlortetracycline from d 7 to 14. A decrease ($P<0.05$) in plasma urea nitrogen and haptoglobin was observed as time increased from d -2 to 14. In summary MCFA have shown to be an effect interventional mitigation strategy against PEDV and various bacteria.

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Approved by:

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will be working with some of them within the industry and know that these individuals will succeed and have a lasting impact on each job they possess.

Dedication

The I would like to dedicate this thesis in honor of my late mother, Vivian Mae Cochrane.

Chapter 1 - Effect of Pelleting on Survival of Porcine Epidemic Diarrhea Virus (PEDV)-Contaminated Feed

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is a heat-sensitive virus that has devastated the United States swine industry. Because of its heat sensitivity, we hypothesized that a steam conditioner and pellet mill mimicking traditional commercial thermal processing may mitigate PEDV infectivity. Pelleting, a common feed processing method, includes the use of steam and shear forces, resulting in increased temperature of the processed feed. Two thermal processing experiments were designed to determine if different pellet mill conditioner retention times and temperatures would impact PEDV quantity and infectivity by analysis of qRT-PCR and bioassay. In Exp. 1, a 3×3×2 factorial design was utilized with 3 pelleting temperatures (68.3, 79.4, and 90.6°C), 3 conditioning times (45, 90, or 180 s), and 2 doses of viral inoculation (low: 1×10^2 Tissue Culture Infectious $Dose_{50}$/g, or high: 1×10^4 Tissue Culture Infectious $Dose_{50}$/g). Non-inoculated and PEDV-inoculated unprocessed mash were used as controls. The low dose PEDV-infected mash had 6.8 ±1.8 cycle time (Ct) greater ($P < 0.05$) PEDV than the high dose mash. Regardless of time or temperature, pelleting reduced ($P < 0.05$) the quantity of detectable viral PEDV RNA compared to the PEDV-inoculated unprocessed mash. Fecal swabs from pigs inoculated with the PEDV-positive unprocessed mash, regardless of dose, were clinically PEDV-positive from 2 to 7 days (end of the trial) post-inoculation. However, if either PEDV dose of inoculated feed was pelleted at any of the 9 tested conditioning time×temperature combinations, no PEDV RNA was detected in fecal swabs or cecum content. Based on Exp. 1 results, a second experiment was developed to determine the impact of lower processing temperatures on PEDV quantity and infectivity. In Exp. 2, PEDV-inoculated feed was pelleted at one of five conditioning temperatures (37.8, 46.1, 54.4, 62.8, 71.1°C) for 30 s. The five increasing processing temperatures led to feed with respective mean Ct values of 32.5, 34.6, 37.0, 36.5, and

36.7 respectively. All samples had detectable PEDV RNA. However, infectivity was only detected by bioassay in pigs from the 37.8 and 46.1°C conditioning temperatures. Exp. 2 results suggest conditioning and pelleting temperatures above 54.4°C could be effective in reducing the quantity and infectivity of PEDV in swine feed. However, additional research is needed to prevent subsequent recontamination after pelleting as it is a point-in-time mitigation step.
Key words: feed, PEDV, pelleting, swine

INTRODUCTION

Recent research has confirmed that porcine epidemic diarrhea virus (PEDV) can be transmitted by swine feed and ingredients (Dee et al., 2014; Schumacher et al., 2015). In a review by Nitikanchana (2014), a theoretical temperature × time relationship was proposed to reduce the infectivity of PEDV in complete feeds based on data extrapolated from PEDV environmental survival studies (Pospischil et al., 2002; Thomas et al., 2014). Typical swine feed pellet mill conditioner retention times and temperatures encompass the theoretical temperature × time relationship proposed. While Goyal et al. (2013) corroborated this relationship using benchtop measures, there is no research confirming this time × temperature relationship using a conditioner and pellet mill that are present in modern feed manufacturing facilities. Although it would be uncommon to set target pellet mill conditioning temperatures below 68°C, it is possible that feed may be produced below these limits during start-up of the pellet mill or during a pellet mill plug. If feed begins to plug in the pellet mill die, the initial attempt to resolve the plug is to turn off steam to the conditioner. This can lead to significant quantities of pelleted feed not reaching the target conditioning temperature. If PEDV particles were in the feed, the resulting feed conditioned at a lower temperature may still have infectious PEDV particles that may potentially contaminate the pellet cooler, post-pellet feed handling equipment, trucks, feed lines,

and feeders at the farm. Therefore, we hypothesize that PEDV dose and pelleting parameters will reduce PEDV quantity and infectivity.

MATERIALS AND METHODS

A corn-soybean meal-based mash swine diet (Table 1.1) was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan, Kansas and used in all experiments. A subsample of this feed was obtained prior to inoculation and confirmed negative by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the presence of PEDV RNA at the Kansas State University Research Park Molecular Diagnostics Development Laboratory in Manhattan, Kansas.

PEDV virus isolate

The mash swine feed was inoculated with the U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338). Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as described by Chen et al. (2014). The stock virus titer contained 4.5 x 10^6 Tissue Culture Infectious Dose $(TCID)_{50}$/ml and was divided into 500 mL aliquots that were stored at -80ºC prior to use, with one aliquot used in each replication.

Experiment 1

Exp. 1 evaluated the role of PEDV dose and varying time × temperature combinations during pelleting on PEDV quantity and infectivity. Treatments were arranged in a 3 × 3 × 2 + 1 + 2 factorial arrangement with 3 pellet mill conditioning temperatures (68.3, 79.4, and 90.6°C), 3 conditioning times (45, 90, 180 sec), and 2 PEDV doses (low dose: $1x10^2$ $TCID_{50}$/g, 20 Ct; and high dose: $1x10^4$ $TCID_{50}$/g, 13 Ct). Two types of controls included a single non-inoculated meal-based negative control and 2 PEDV-inoculated meal-based positive controls that were not thermally processed (one at each dose). The PEDV dosages were selected based on the known

minimum infectious dose of PEDV in swine feed determined by Schumacher et al. (2015). Pellet mill conditioning temperatures and times were selected based on traditional industry parameters for pellet quality and extreme parameters for hygienic pelleting to mitigate other biological hazards, such as *Salmonella* spp. (Cochrane et al., 2015).

PEDV Inoculum

After mixing the basal diet, negative control mash samples were collected. Next, a feed inoculum was created by mixing 500 mL aliquot of the stock virus into a 4.5 kg batch of feed. The feed and virus were mixed using a bench-top laboratory scale stainless steel paddle mixer (Cabela's Inc, Sidney, NE.) for a total of 2.5 min with one rotation of the paddles per second.

The above procedures were then repeated for the high dose inoculation. Samples of each mash diet were aseptically collected as the positive low dose and high dose feed controls prior to pelleting.

Thermal processing

The low and high dose inoculated feeds were thermally processed using a pilot-scale single pass conditioner and pellet mill (Model CL5, CPM, Waterloo, IA). Prior to pelleting the first treatment, non-inoculated feed was processed until the exit temperature of the feed was stable at the target temperature. To help reduce cross-contamination, the low dose batches were pelleted prior to the high dose batches. During thermal processing, within each dose, the treatment with the lowest temperature and longest retention time were pelleted first. The temperature was then held constant until the other two retention times were achieved and samples were collected. This process was then repeated at the two higher temperatures. The temperature for each treatment was measured at the discharge from the conditioner to the pellet die feeder screw. Once the low dose treatments were completed, the same procedure was used to pellet the high dose treatments.

For each temperature, time, and dose combinations, 3 pelleted samples were collected for qRT-PCR and bioassay analysis. In addition, between each inoculated batch, a minimum of 5 kg of virus-free feed was processed through the pellet mill. This was done to prevent virus carry over between treatments and to stabilize the conditioning temperature to ensure the contaminated feed was processed under uniform temperature conditions.

Samples preparation and storage

Three 100 g samples of each batch of feed were added to 400 ml of cold phosphate buffered saline (PBS, Life Technologies; pH, 7.4) in 500 ml bottles (Nalgene square bottles; Thermo Scientific, Waltham, MA), and thoroughly mixed and stored at 4°C for approximately 12 h. A 2 mL aliquot of feed suspension was then evaluated using a PEDV N-gene based qRT-PCR at Kansas State University. A 20 mL aliquot was also harvested and frozen at -80°C for use in the bioassay.

Bioassay

The Iowa State University Institutional Animal Care and Use Committee reviewed and approved the pig bioassay protocol. A total of 63 pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. Additionally, all pigs were confirmed negative for PEDV, porcine delta coronavirus (PDCoV) and transmissible gastroenteritis virus (TGEV) based on fecal swab. To further confirm PEDV-negative status, collected blood serum was analyzed for PEDV antibodies by an indirect fluorescent antibody (IFA) assay and TGEV antibodies by ELISA, both conducted at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). Pigs were allowed 2 d of adjustment to the new pens before the bioassay began.

A total of 21 rooms (63 pigs, 3 per room) were assigned to treatment groups with 1 negative control room, 2 positive control rooms (low and high dose), and 18 rooms representing treatment diets (3 conditioning temperatures × 3 conditioning times × 2 PEDV dose levels). Each of the 3 pigs in each room received a separate feed sample of the experimental diet via oral gavage. For example, feed samples inoculated with the low dose of PEDV ($1x10^2$ $TCID_{50}$/g, 20 Ct) and conditioned at 68.3°C for 45 s were fed to 1 room. Within that room, each pig was inoculated with a different feed sample from the experimental treatment. This resulted in 3 pigs per time×temperature×dose treatment.

During the bioassay, rectal swabs were collected on d 0, 2, 4, 6, and 7 post inoculation (dpi) from all pigs and tested for PEDV RNA qRT-PCR. Following humane euthanisia at 7 dpi, small intestine, cecum, and colon samples were collected at necropsy along with an aliquot of cecal contents. One section of formalin-fixed proximal, middle, distal jejunum and ileum was collected per pig for histopathology and Immunohistochemistry (IHC) (Chen et al., 2014).

Experiment 2

Based on the results of Exp. 1, a second study was designed in order to evaluate the effect of increasing pellet mill temperatures on PEDV inactivation. The experiment was designed to mimic a real life situation that could occur in the feed mill, which is a pellet mill plug. In this situation the feed would become lodged in the pellet mill die and the steam would be turned off in order to free the material within the die. This situation leads to feed not reaching the target pellet temperature. Therefore, the purpose of Exp. 2 was to evaluate 5 increasing conditioner temperatures ranging from 37 to 71°C on PEDV inactivation if a plug were to occur within the pellet mill.

PEDV Inoculum

Exp. 2 was carried out in the Cargill Feed Safety Research Center at Kansas State University. In order to accomplish the objective for Exp. 2, PEDV-free feed was first collected, served severed as the PEDV negative control, and was used to make the PEDV inoculum. Only the high dose PEDV stock virus ($1x10^4$ $TCID_{50}$/g, 16 CT) was used for Exp. 2. An inoculum premix was created by mixing 500 mL aliquot of the stock virus into a 4.5 kg batch of feed of swine feed using procedures established in Exp. 1. The feed and inoculum were mixed using a bench-top laboratory scale stainless steel paddle mixer (Cabela's Inc, Sidney, NE) for a total of 2.5 min with one rotation of the paddles per second.

The PEDV feed inoculum (4.5 kg of feed + 500 ml of stock virus) was then added to 45 kg of PEDV-free swine diet to form the positive control in a 0.11 m^3 electric paddle mixer (H. C. Davis Sons Manufacturing model# SS-L1; Bonner Springs, KS). The entire batch was then mixed for 5 min creating the PEDV positive control, discharged for 10 min into biohazard containers, and finally held at -2°C, for approximately one hour, until thermal processing.

Thermal processing

Porcine epidemic diarrhea virus-free feed was pelleted using the same pilot-scale single pass conditioner and pellet mill (Model CL5, CPM, Waterloo, IA) used in Exp. 1. In order to mimic a plug within the pelleting system, the pellet mill was heated by pelleting PEDV-free feed with a conditioning temperature of 71°C which would represent normal production temperature. The pellet mill was heated to 71°C for 60 min in order to have the entire pellet mill reach optimum temperature. Temperature was again measured by a thermometer at the discharge from the conditioner to the pellet die feeder screw. At 60 min, the steam valve was then turned off until the conditioning temperature dropped below 37°C to mimic procedures commonly used to resolve a plug in the conditioner or pellet die. Next, PEDV-inoculated feed was placed into the

pellet mill hopper. Once PEDV-inoculated feed started passing through the pellet mill, steam was slowly added, and five pelleted samples were collected at targeted hot mash conditioner temperatures of 37, 46, 54, 62, and 71°C (± 1.2°C) using a 30 sec conditioning time. These conditioning temperatures were selected based on a previously-determined prediction equation for the specific pellet mill.

Exp. 2 was carried out three separate times (days) within the feed safety research center with complete decontamination of the facility between each run in order to create three true replications.

Samples preparation and storage

Three 100 g samples of each batch of feed were added to 400 ml of cold PBS (Life Technologies; pH, 7.4) in 500 ml bottles (Nalgene square bottles; Thermo Scientific, Waltham, MA), and thoroughly mixed and stored at 4°C for approximately 12 h. A 2 mL aliquot of feed suspension was then evaluated using a PEDV N-gene based qRT-PCR at Kansas State University. A 20 mL aliquot was also harvested and frozen at -80°C for use in the bioassay.

Bioassay

A total of 48 pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. Procedures and evaluation for the bioassay were carried out as described in Exp. 1 bioassay.

A total of 16 rooms (48 pigs, 3 per room) were assigned to treatment groups with 1 negative control room and 15 rooms representing treatment diets (5 conditioning temperatures × 3 replicates/temperature). Each of the 3 pigs in each room received a separate feed sample representing 1 of 3 processing days of the experimental diet via oral gavage, For example, feed samples conditioned at 37°C were fed to 3 different rooms. Within each room, Pig 1 received the

sample manufactured on Thermal Processing Day 1, Pig 2 received the sample manufactured on Thermal Processing Day 2, and Pig 3 received the sample manufactured on Processing Day 3. This resulted in 9 pigs per conditioning temperature.

Each pig from the negative control room was given a 10 mL aliquot of inoculum created from the 0.11 m^3 electric paddle mixer. Different from the negative control room, each pig in each challenge room was given an aliquot of inoculum from the replicate, temperature and processing day, resulting in three samples of the same temperature treatment and different processing days. One room was representative of a treatment with three rooms per treatment. Rectal swabs and intestinal sections were evaluated in the same fashion as the Exp. 1 bioassay.

Statistical Analysis

Statistical analysis was carried out using SAS version 9.4 (SAS Institute, Inc., Cary, NC). In Exp 1, the PROC MIXED procedure of SAS (SAS Institute, Inc., Cary, NC) was used to evaluate PEDV RNA feed Ct values, villus height, crypt depth, villous height to crypt depth ratio, and immunohistochemistry. Fixed effects included temperature, time, dose, and their combination. In Exp. 2, data of the effects of conditioner temperature on feed CT values, villus height, crypt depth, and villus height to crypt depth ratio were analyzed as a completely randomized design using PROC GLIMMIX in SAS (SAS Institute, Inc., Cary, NC) with pig as the experimental unit by a pairwise comparison. Treatment was the fixed effect. Results for treatment criteria were considered significant at $P \leq 0.05$ and marginally significant from $P > 0.05$ to $P \leq 0.10$.

RESULTS

Experiment 1

There was no PEDV RNA detected in the unprocessed PEDV-free feed. When the low-dose PEDV (Ct 20) was mixed with the feed, the resulting feed Ct value was 31, and when the high

PEDV dose media (Ct 13) was mixed with feed the resulting Ct value was 24 (Table 1.2). The lose dose processed treatments ranged from 36 to 45 CT compared to the high dose processed treatments of either 30 or 31 CT (Table 1.2).

As expected, fecal shedding of PEDV was not detected in rectal swabs from negative control pigs for the duration of the study (Table 1.2). Fecal swabs from pigs fed the low-and high-PEDV dose positive control treatment (inoculated, but non-processed feed) were PEDV-positive from 2 dpi through the end of the study at 7 dpi. Cecum contents at 7 dpi and IHC determined 7 dpi were also positive for the positive control pigs (Table 1.2 and 1.3). However, if either the low-or high-dose PEDV feed was processed at any of the 9 possible conditioning time × temperature combinations, no PEDV RNA was detected in fecal swabs or cecum contents at 7 dpi.

The villous height for pigs challenged with the non-inoculated feed was higher ($P < 0.05$) compared to the height in pigs challenged with the high dose PEDV unprocessed feed (Table 3). Porcine epidemic diarrhea virus IHC immunoreactivity was not visible in the cytoplasm of villous enterocytes of low or high dose challenged pigs from any of the time and temperature pellet treatment combination for the duration of the study. However, immunoreactivity was detected within the low-and high-PEDV dose positive control treatment (inoculated, but non-processed feed) (Table 1.3).

Experiment 2

When PEDV-inoculated feed was processed at five different conditioning temperatures (37, 46, 54, 62, and 71°C), the respective mean cycle threshold (Ct) values as detected by qRT-PCR were 32.5, 34.6, 37.0, 36.5, and 36.7, respectively (Table 1.4). All 9 of the feed samples conditioned at 37, 46, or 54°C had detectible PEDV RNA; whereas 8 of the 9 processed at 62, and 71°C had

detectible PEDV RNA. It was observed that Ct value increased as conditioning temperature increased to 54°C, with little change thereafter.

As in Exp. 1, fecal shedding of PEDV was not detected in rectal swabs or cecum contents from pigs fed the PEDV-negative control for the duration of the study (Table 1.4). Of the 9 total pigs gavaged with aliquots from the PEDV-positive diet conditioned at 37°C, a fecal swab from 1 pig (Room 7, Replicate 2) yielded detectible PEDV RNA at 2 dpi, and all 3 pigs in Room 7 had fecal swabs and cecum contents with detectible viral particles by 4 through 7 dpi. In addition, 3 pigs gavaged with aliquots from the treatment conditioned at 46°C had detectible fecal PEDV RNA at 2 to 7 dpi, and all pigs were in the same room (Room 8, Replicate 2). No pig challenged with feed conditioned at or above 54°C had detectible PEDV RNA in fecal swabs or cecum content for the duration of the study.

The pigs challenged with the feed conditioned at 37°C had shorter ($P < 0.05$) villous heights than pigs challenged with any other temperature treatment (Table 1.5). Furthermore, pigs challenged with feed conditioned at 46 or 71°C had different ($P < 0.05$) crypt depths when compared to one another but were statistically similar to all other treatments (Table 1.5). This led to pigs challenged with feed conditioned at 37°C to have the lowest villus height: crypt depth ratio followed increasing ratios as temperature increased (Table 1.5). Porcine epidemic diarrhea virus IHC immunoreactivity was visible in the cytoplasm of villous enterocytes of pigs challenged with the two lowest processed temperatures, 37 and 46°C, when harvested at 7 dpi.

DISCUSSION

Temperature and time studies have been carried out on PEDV in feces, feed, and ingredients. However, some of those temperatures are not applicable to the feed and ingredient industries. Fecal material has been tested at 71°C for 10 minutes, 63°C for 10 minutes, 54°C for 10 minutes,

38°C for 12 hours, 20°C for 24 hours, and 20°C for 7 days on metal surfaces (Thomas et al., 2015). From these time × temperature combinations, only the 71°C for 10 minutes and 20°C for 7 days led to bioassay negative pigs (Thomas et al., 2015). However, all of the other treatments led to infection in pigs (Thomas et al., 2015). When comparing the temperatures used to the present study, the first issue that arises is that the 54 °C treatments differed in the bioassay results. In this study all pigs were bioassay negative when treated at 54 °C. This could be due to the nature of the pellet mill as steam is directly being added to the feed and then the feed is forced through a pellet die. As the feed is forced through the die it could also undergo frictional heat from being pushed through the small diameter holes. However, in both trials in which 71°C was reached, it led to PEDV negative bioassay regardless of the treatment time.

PEDV has also been shown to survive up to seven days in fresh feces at 30, 50, and 70% relative humidity in combination with 40, 50, 60°C, and at room temperature for 14 days when placed into a slurry (Goyal, 2013). The major difference between the data from the fecal material and the present feed study is that the use of a pellet mill inactivates PEDV at a much lower temperature (54.4°C) compared to an incubator (71°C).

Ingredients and complete feed have been evaluated at 60, 70, 80, or 90°C for 0, 5, 10, 15, or 30 minutes (Trudeau et al., 2015). In each instance there was no difference between the ingredients that were heated for 30 minutes (Trudeau et al., 2015). There was also no detectable virus after 30 minutes of heating at 90°C (Trudeau et al., 2015). However, these samples were placed in Vero-81 cells and not directly in to pigs. In a separate study time, temperature, and relative humidity were evaluated. In that study 99.99% of PEDV is inactivated by heating at 90°C and 70% relative humidity for 10 minutes (Goyal, 2014). A temperature of 90°C in both studies and in the current study all resulted in a negative result.

More extreme temperatures have also been evaluated in which 145°C inactivated PEDV after 10 minutes (Trudeau et al., 2016). When comparing the previous studies to the present study the use of a pellet mill inactivated PEDV at a faster rate (30 sec) and much lower temperature (54.4°C). This could be due to the heat source and the frictional heat that occurs as the pellet exits the pellet die.

Another commercial process that is used for some ingredients in the feed industry, including plasma, is the use of spray drying. This process uses high temperatures throughout the manufacturing process, which makes it an option to test against PEDV. In a study by Gerber et al., inlet temperatures of 166°C and outlet temperature of 80°C were used in which these conditions led to the inactivation of infectious PEDV (2014). In a separate study, bovine plasma was subjected to the spray drying process in which the inlet temperature was 200°C and either 70 or 80°C throughout the outlet (Pujols, 2014). The bovine plasma subjected to spray drying was not infectious after the process (Pujols, 2014). However, after the spray drying process the plasma was re-inoculated with PEDV and subjected to storage times of 7, 14, or 21 and temperatures of 4, 12, or 22°C (Pujols, 2014). The virus was not infectious at any of the time points at 22°C, but was infectious in 1 out of 5 samples in 12°C at 7 days, and 4 out of 5 samples in 4°C at 7 days (Pujols, 2014). The initial high temperatures which were greater than those used in this study lead to the inactivation of the virus, however the low storage temperatures of 4 and 12°C did not inactivate the virus. This is extremely important to note since manufacturing equipment requires start up time and in some instances, as shown in the present study, the heat source turned off to clear lodged material. This in turn could potentially lead to contamination of material later in processing and transportation. From this study it was deemed that a temperature of 54.4°C or higher is required inactivate PEDV in complete feed when using a pellet mill and

placing the feed into a swine bioassay. It is important to point out a few limitations of the two studies. The first is that the studies were carried out on a pilot size pellet mill. Every pellet mill will operate differently depending on the size, steam addition, time, and material flowing through the process. The second issue is that the heating and cooling time of pellets. For this study pellets were immediately placed on ice to cool them. If a pellet mill cooler would have been used, the cooling time would have been longer in which the pellets would have maintained their internal heat for a longer time period. The third limitation is the use of a bioassay. Currently a bioassay is the best diagnostic method to measure infectivity. However, as explained by Davies (2015), the risk of an individual pig becoming infected is very low, but carries a high collective risk. In the bioassay pigs are only receiving 10mL of a sample compared to their normal feed consumptions. If larger amounts of the pelleted feed were given to the pigs, then the likelihood of infection would have increased in the study. However, research generated using the same feed and pig bioassay model has led to infection within the animals (Schumacher et al, 2015; Cochrane et al., 2016). Because of the issues associated with this study, further research needs to be carried out on a larger scale looking at the use of different pellet mills and pellet cooling time.

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TABLES AND FIGURES

Table 1.1 Diet composition used in Exp. 1 and 2

Item	Negative control
Ingredient, %	
Corn	79.30
Soybean meal, 46.5 CP	15.70
Choice white grease	1.00
Monocalcium phosphate	1.40
Limestone, ground	1.15
Salt	0.50
L-Threonine	0.03
Trace mineral premix[1]	0.15
Sow add pack[2]	0.50
Vitamin premix[3]	0.25
Phytase[4]	0.02
Total	100.00
Formulated analysis, %	
Dry matter	91.4
Crude protein	17.1
Crude fiber	3.7
Ether extract	3.5
Ca	0.78
P	0.52

[1]Each kilogram contains 26.4 g Mn, 110 g Fe, 110 g Zn, 11g Cu, 198 mg I, and 198 mg Se.
[2]Each kilogram contains 220,000 mg choline, 88 mg biotin, 660 mg folic acid, 1,980 mg pyridoxine.
[3]Each kilogram contains 4,400,000 IU vitamin A, 660,000 IU vitamin D_3, 17,600 IU vitamin E, 1,760 mg menadione, 3,300 mg riboflavin, 11,000 mg pantothenic acid, 19,800 mg niacin, 15.4 mg vitamin B_{12}.
[4]High Phos 2700 GT, DSM Nutritional Products, Parsippany, NJ.

Table 1.2 Effects of porcine epidemic diarrhea virus (PEDV) dose, pelleting temperature, and conditioning retention time on PEDV detection from feed, pig fecal swabs and cecum contents, Exp. 1

		PEDV N-gene qRT-PCR, cycle threshold (Ct)[1]						
				Fecal swabs				Cecum contents
PEDV dose, temp.[3], and time[4]	Tissue culture (Ct)	Feed	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi
Unprocessed virus-free feed[5]		45.0	- - -[8]	- - -	- - -	- - -	- - -	- - -
Low dose inoculated feed[6]	20.0	30.7^{e}	- - -	22.4	18.2	18.8	24.1	26.7
68.3°C 45s		42.6^{ab}	- - -	- - -	- - -	- - -	- - -	- - -
68.3°C 90s		39.5^{bcd}	- - -	- - -	- - -	- - -	- - -	- - -
68.3°C 180s		45.0^{a}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 45s		36.7^{cd}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 90s		39.7^{bc}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 180s		42.3^{ab}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 45s		39.7^{bc}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 90s		37.4^{cd}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 180s		35.9^{d}	- - -	- - -	- - -	- - -	- - -	- - -
High dose inoculated feed[7]	13.0	23.9^{f}	- - -	23.0	15.3	20.4	24.3	24.0
68.3°C 45s		30.2^{e}	- - -	- - -	- - -	- - -	- - -	- - -
68.3°C 90s		29.7^{e}	- - -	- - -	- - -	- - -	- - -	- - -
68.3°C 180s		30.2^{e}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 45s		30.1^{e}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 90s		29.5^{e}	- - -	- - -	- - -	- - -	- - -	- - -
79.4°C 180s		30.2^{e}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 45s		30.1^{e}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 90s		30.6^{e}	- - -	- - -	- - -	- - -	- - -	- - -
90.6°C 180s		30.0^{e}	- - -	- - -	- - -	- - -	- - -	- - -
	SEM	1.27						

[1]An initial tissue culture containing a low dose and high dose of PEDV was used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per treatment (10 ml per pig). Thus, each value represents the mean of 3 pigs per treatment. Pigs were initially 10 d old and 3.6 kg BW.

[2]Day post inoculation.

[3]Temperature of feed exiting the conditioner.

[4]Retention time. The amount of time required for feed to pass through the conditioner.

[5]A cycle threshold (Ct) of >45 was considered negative for presence of PEDV RNA.

[6]For low dose feed, PEDV (1×10^3 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^2 $TCID_{50}$/g of feed.

[7]For high dose feed, PEDV (1×10^5 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^4 $TCID_{50}$/g of feed.

[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

[8] In each instance a (–) signals a negative pig in the bioassay and a (+) represents a positive fecal swab in the bioassay. Each day post inoculation within each treatment has three symbols with each row and column which represents one of the three pigs in each treatment.

Table 1.3 Effects of porcine epidemic diarrhea virus (PEDV) dose, pelleting temperature, and conditioning retention time on morphologic and immunohistochemistry evaluation of small intestine from pigs, Exp. 1

Item[1]		Morphology[2]			
		Villus height, μm	Crypt depth, μm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[3]
Unprocessed virus-free feed		463.4^{abcd}	112.5	4.2^{abcde}	0^{b}
Low dose inoculated feed[4]		414.3^{de}	91.0	4.6^{abcd}	0.3^{b}
68.3°C	45s	481.6^{abcd}	101.3	4.8^{abc}	0^{b}
	90s	489.3^{abcd}	108.1	4.6^{abcd}	0^{b}
	180s	504.4^{abc}	115.6	4.4^{abcd}	0^{b}
79.4°C	45s	508.6^{ab}	108.9	4.7^{abcd}	0^{b}
	90s	476.4^{abdc}	103.6	4.6^{abcd}	0^{b}
	180s	460.6^{abcde}	93.1	4.9^{ab}	0^{b}
90.6°C	45s	443.2^{abcde}	97.8	4.6^{abcd}	0^{b}
	90s	514.5^{a}	103.3	5.0^{a}	0^{b}
	180s	441.8^{abcde}	103.6	4.3^{abcde}	0^{b}
High dose inoculated feed[7]		309.3^{f}	112.6	3.1^{f}	1.7^{a}
68.3°C	45s	423.1^{cde}	105.3	4.0^{abcdef}	0^{b}
	90s	429.4^{bcde}	118.3	3.7^{def}	0^{b}
	180s	389.2^{ef}	100.0	4.0^{bcdef}	0^{b}
79.4°C	45s	432.8^{abcde}	117.3	3.7^{cdef}	0^{b}
	90s	390.6^{ef}	102.5	3.7^{cdef}	0^{b}
	180s	448.7^{abcde}	104.5	4.3^{abcd}	0^{b}
90.6°C	45s	383.1^{ef}	119.5	3.2^{ef}	0^{b}
	90s	446.4^{abcde}	102.8	4.4^{abcd}	0^{b}
	180s	408.9^{de}	105.7	3.9^{bcdef}	0^{b}
	SEM	29	10.2	0.4	0.2

[1]An initial tissue culture containing a low dose or high dose of PEDV was used to inoculate batches of feed. Three feed samples per batch were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per treatment (10 ml per pig). Thus, each value represents the mean of 3 pigs per treatment necropsied at 7 d post infection. Pigs were initially 10 d old and 73.6 kg BW.

[2]Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin for evaluation.

[3]Three sections of ileum were evaluated and averaged into one categorical value per pig. Categorical values were assigned for each pig (0=no signal, 1=mild, 2=moderate, 3=abundant, 4=diffuse) and reported as the mean from 3 pigs per treatment, thus the mean of 9 values.

[4]For low dose feed, PEDV (1×10^3 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^2 $TCID_{50}$/g of feed.

[5]Temperature of feed exiting the conditioner.

[6]Retention time. The time feed was inside the conditioner.

[7]For high dose feed, PEDV (1×10^5 $TCID_{50}$/ml) was diluted into feed to provide a dose of 1×10^4 $TCID_{50}$/g of feed.

[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

Table 1.4 Influence of processed porcine epidemic diarrhea virus (PEDV) inoculated feed on qRT-PCR cycle threshold (Ct) of feed, fecal swabs and cecum contents of pigs Exp. 2

		Fecal swabs, Ct					
Item[1]	KSU feed inoculum, Ct	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi Cecum content, Ct
Processed feed, % positive[3]							
Negative	-[4]	-	-	-	-	-	-
37.8°C	100.0 (9/9)	-	(1/9)	(3/9)	(3/9)	(3/9)	(3/9)
46.1°C	100.0 (9/9)	-	(3/9)	(3/9)	(3/9)	(3/9)	(3/9)
54.4°C	100.0 (9/9)	-	-	-	-	-	-
62.8°C	88.9 (8/9)	-	-	-	-	-	-
71.1°C	88.9 (8/9)	-	-	-	-	-	-
Processed feed, Ct values[5]							
Negative	-	-	-	-	-	-	-
37.8°C	32.5[a]	-	15.8	27.5	16.6	17.8	16.9
46.1°C	34.6[b]	-	24.5	15.2	15.4	17.9	18.8
54.4°C	37.0[c]	-	-	-	-	-	-
62.8°C	36.5[c]	-	-	-	-	-	-
71.1°C	36.7[c]	-	-	-	-	-	-

[1]500 ml of tissue culture containing 4.5×10^6 $TCID_{50}$/ml of PEDV was inoculated into a 4.5 kg batch of feed, then added to 45 kg of PEDV negative feed to form the positive treatment. One feed sample per temperature per replicate × 3 replicates was collected, divided into three aliquots and diluted in PBS to form supernatants. Thus each feed supernatant value per treatment represents the mean of 3 replicates × 3 repetitions.

[2]Day post inoculation.

[3]Means represent the percent of samples that had detectible RNA by PEDV qRT-PCR analysis (< 45 Ct).

[4]No detectible PEDV RNA (Ct >45).

[5]Mean cycle threshold (Ct) value of samples with detectible PEDV RNA below 45.

[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

Table 1.5 Morphologic and immunohistochemistry evaluation of small intestine from pigs that were challenged with porcine epidemic diarrhea virus (PEDV) inoculated feed processed at increasing temperatures Exp. 2

	Morphology[2]			
Item[1]	Villus height, μm	Crypt depth, μm	Villus height to crypt depth ratio	Immunohistochemistry (IHC)[3]
Processed feed				
Negative	365.1±34.9^{ab}	190.7±17.3^{ab}	1.9±0.2^{abc}	0
37.8°C	299.9±20.1^{b}	192.4±10.0^{ab}	1.6±0.1^{ac}	0.7
46.1°C	394.2±20.1^{a}	230.±10.0^{a}	1.7±0.1^{bc}	0.6
54.4°C	393.9±20.1^{a}	200.0±10.0^{ab}	2.0±0.1^{ab}	0
62.8°C	405.1±20.1^{a}	206.8±10.0^{ab}	2.0±0.1^{ab}	0
71.1°C	420.9±20.1^{a}	184.1±10.0^{b}	2.3±0.1^{a}	0

[1]500 ml of tissue culture containing 4.5×10^6 $TCID_{50}$/ml of PEDV was inoculated into a 4.5 kg batch of feed, then added to 45 kg of PEDV negative feed to form the positive treatment. One feed sample per temperature per replicate × 3 replicates was collected, divided into three aliquots and diluted in PBS to form supernatants. Thus each feed supernatant value per treatment represents the mean of 3 replicates × 3 repetitions.
[2]Intestinal cross-sections were fixed in formalin and stained with hematoxylin and eosin (H&E) for evaluation.
[3]Three sections of ileum were evaluated and averaged into one categorical value per pig. Categorical values were assigned for each pig (0=no signal, 1=mild, 2=moderate, 3=abundant, 4=diffuse) and reported as the mean from 3 pigs per negative treatment and mean from 9 pigs per remaining treatments.
[a,b]Means within column lacking a common superscript are different ($P < 0.05$).

Chapter 2 - Evaluating mitigation additives on Porcine Epidemic Diarrhea virus (PEDV) in swine feed and ingredients

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ABSTRACT

Research has confirmed contaminated swine feed or ingredients as potential vectors of Porcine Epidemic Diarrhea Virus transmission. Therefore, two experiments were conducted to evaluate the effectiveness of various mitigation additives to prevent or mitigate PEDV post-processing contamination in swine feed and ingredients. In Exp. 1, treatments were arranged in a 7×4×7 factorial with 7 mitigation treatments, 4 feed matrices, and 7 analysis days. Formaldehyde, medium chain fatty acids (MCFA), essential oils (EO), and organic acid (OA) addition each decreased detectable PEDV RNA compared to the control ($P < 0.05$). Additionally, PEDV stability over time was influenced by matrix as the meat and bone meal and spray-dried animal plasma resulted in a greater quantity of detectable PEDV RNA over 42 days compared to that of the swine diet and blood meal ($P < 0.05$). In Exp. 2, a 4×2×7 plus 2 factorial was utilized with 4 mitigation treatments, 2 feed matrices, and 7 analysis days plus 1 treatment each of PEDV negative untreated feed and plasma. Feed treated with MCFA, regardless of inclusion level, had lower ($P < 0.05$) RNA concentration than feed treated with formaldehyde. The SDAP treated with formaldehyde had lower ($P < 0.05$) RNA concentrations than untreated SDAP, which had similar ($P > 0.05$) levels as SDAP-treated with either 1% or 2% MCFA. In bioassay, the 1% MCFA inclusion was equally effective at mitigating PEDV as a commercially available formaldehyde product in the complete swine diet. In summary, time, formaldehyde, MCFA, EO, and OA all enhance the RNA degradation of PEDV in swine feed and ingredients, but their effectiveness varies within matrix. It was also demonstrated that a 1% inclusion of MCFA was as effective as a commercial formaldehyde based product in a complete swine diet at preventing PEDV post-processing infection within a swine bioassay.

INTRODUCTION

Porcine Epidemic Diarrhea Virus (PEDV) is an enveloped single-stranded positive-sense RNA virus that was first identified in the United States in May 2013 [1, 2]. The virus is known to primarily be spread by the fecal-oral route, but epidemiological and controlled experiments confirm that complete feed or feed components can be one of the many possible vectors of transmission of PEDV [1, 3, 4, 5]. Viral transmission via feed may be by direct contamination, but is more likely from cross-contamination during the manufacturing, transportation, and storage of feed and ingredients [6]. Viral destruction by thermal processing has been evaluated, but is a point-in-time strategy that does not offer residual protection from contamination post-processing, which is a solution offered by mitigation alternatives [7].

Mitigation additives, such as formaldehyde, are effective at mitigating *Salmonella* in animal feed, and research suggests it is also effective against PEDV [5, 8, 9,10]. However, formaldehyde does not have regulatory approval for PEDV mitigation in the United States [11], requires specialized equipment for successful application, carries potential worker health concerns, and may be perceived negatively by consumers [12]. Medium chain fatty acids are also effective at mitigating enveloped viruses and bacteria, but the concentrations that are required can be quite high [13,14]. Organic acids have been studied as an antimicrobial agent for several decades, and have been shown to effectively mitigate bacterial and some extremely detrimental viruses, such foot and mouth disease and African swine fever [14,15,16]. However, the knowledge of effectiveness of organic acids against other viruses, such as PEDV, is limited. Essential oils have also showed antimicrobiala as well as antiviral RNA effects [17]. Sodium bisulfate is a commercial product, which is used in the broiler and pet food industry for microbial control, particularly against *Salmonella* spp. [18]. It has not been evaluated against viruses or for

use in the swine industry, but its desiccant and acidulant properties warrant evaluation for effectiveness against PEDV. Sodium bisulfate could also be of interest because of its dry powder form and easy implementation by the feed industry compared to liquid alternatives [18]. Finally, sodium chlorate is effective at pathogen mitigation when included in drinking water of livestock [19] and for PEDV mitigation of surfaces [20]. Because of various physical states, chemical composition, and electrostatic properties of each additive and feed matrix, the overall effectiveness as a mitigant may be different. Therefore, the objective of this experiment was 2-fold: 1) evaluate the effectiveness of various mitigation additives post-processing PEDV contamination in swine feed and feed ingredients by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) and 2) further evaluate the mitigation additives, that were deemed to be effective against PEDV based on the objective 1 RT-qPCR results in a 10-d old swine bioassay.

MATERIALS AND METHODS

Experiment 1

Treatment application

In experiment 1, a 7×4×7 factorial was utilized with 7 mitigant treatments, 4 feed matrices, and 7 analysis days. The treatments included: 1) negative control with no treatment addition, 2) 0.3% commercial formaldehyde based product (Termin-8, Antiox Corp, Lawrenceville, GA), 3) 1% sodium bisulfate (SBS; Jones-Hamilton Co, Walbridge, OH), 4) 1% sodium chlorate, 5) 3% organic acid blend [OA; lactic, propionic, formic, and benzoic; 1:1:1; Sigma Aldrich, St. Louis, MO], 6) 2% essential oil blend [EO; garlic oleoresin, turmeric oleoresin, capsicum oleoresin, rosemary extract, and wild oregano essential oils], and 7) 2% medium chain fatty acid blend [MCFA; caproic, caprylic, and capric acids; 1:1:1; Sigma Aldrich,

St. Louis, MO]. The 4 matrices included: 1) complete swine diet, 2) blood meal, 3) porcine meat and bone meal, and 4) spray-dried porcine plasma. The 7 analysis days included d 0, 1, 3, 7, 14, 21, and 42 post inoculation. None of the matrices had previous mitigants added and were tested for proximate analysis, fatty acid content, and amino acid content (Tables 2.1.-2.3). The complete swine diet was a Phase 3 swine nursery diet manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center, Manhattan, KS. All protein meals were obtained in dried form and untreated with preservatives, antimicrobials, or other additives. The avian blood meal and porcine meat and bone meal were obtained from Valley Proteins, Inc., (Winchester, VA) and the spray-dried porcine plasma from a third-party distributor (manufactured by American Proteins, Cumming, GA). All feed matrices tested negative for PEDV by RT-qPCR prior to the experiment. One kilogram (kg) of each feed matrix was placed in a lab scale ribbon mixer where the liquid treatments were aerosolized onto the feed and the solid treatments were mixed directly into the mixer. All treatments were applied on a wt/wt basis. The dry powder treatments were mixed for 3 minutes, the EO treatment mixed for 15 minutes because of the known viscosity of the product, and all other liquid treatments were mixed for 5 minutes. Once the treatments were mixed, a total of 90 g of product (9 g collected from 10 different locations) was placed into a polyethylene container for inoculation.

Between treating the different matrices with the same treatment, the mixer was physically cleaned to remove all organic residue. Between different treatments, the mixer was physically and wet cleaned and dried to remove all residues. A ground corn flush was also used between treatments to prevent treatment-to-treatment cross-contamination.

PEDV Virus isolate

The U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 7 (PEDV19338) was used to inoculate feed. Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as described by Chen et al. [2]. The stock virus titer contained 5.6 x 10^5 $TCID_{50}$/ml.

Inoculation

The 28 samples (7 mitgant treatments × 4 feed matrices) were inoculated in polyethylene containers at the Kansas State University Veterinarian Diagnostic Laboratory. A total of 10 mL (1 ml cell fluid + 9 ml tissue culture medium) was added to each 90-g sample to result in 100 g of inoculated feed matrix. The 10 mL inoculum was added by two 5 mL additions, and the container was sealed and shaken to distribute virus after each addition. Each of the 28 inoculated samples were divided into twenty-one 3-g sub-samples by analysis day and placed into 15 mL conical tubes. The samples were then stored at ambient temperature until aliquoted for viral RNA expression of PEDV at d 0, 1, 3, 7, 14, 21, and 42 days post treatment via RT-qPCR. There were three replicates per sub-sample. Supernatant from the untreated controls for each of the four matrices on d 0 was harvested and aliquots frozen to use as controls or each subsequent day's analysis to determine intra- and inter-assay variation. There was very little variation among sampling day or within duplicate, suggesting that the RT-qPCR assay was highly sensitive, accurate, and precise (Table 2.4).

Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)

Twelve milliliters (mL) of 1× PBS (Life Technologies, Grand Island, NY) was added to each 3-g sample, vortexed and placed in a 4°C refrigerator overnight. The following day, 1 mL of supernatant was removed for archiving. Fifty microliters (μL) of supernatant from each sample were loaded into a deep well plate and extracted using a Kingfisher 96 magnetic particle

processor (Fisher Scientific, Pittsburgh, PA) and the MagMAX-96 Viral RNA Isolation kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions with one modification, reducing the final elution volume to 60 µL. One negative extraction control consisting of all reagents except sample was included in each extraction, as well as two replicates of an aliquot of the Day 0 untreated controls for all sample types. The extracted RNA was frozen at -20°C until assayed by RT-qPCR. Analyzed values represent cycle threshold (Ct) at which the virus was detected, and thus lower values indicate greater nucleic acid presence, not infectivity.

A duplex RT-qPCR was designed for the dual purpose of detecting porcine epidemic diarrhea virus (PEDV) in samples by targeting the nucleocapsid, and monitoring extraction efficiency by targeting the 18S ribosomal RNA subunit. Primers and probes for PEDV and 18S (PEDVn-F2: GCT ATG CTC AGA TCG CCA GT, PEDVn-R2: TCT CGT AAG AGT CCG CTA GCT C, PEDVn-Pr2 probe: FAM-TGC TCT TTG GTG GTA ATG TGG C-BHQ1, and 18S-F: GGA GTA TGG TTG CAA AGC TGA, 18S-R: GGT GAG GTT TCC CGT GTT G, 18S-Pr probe: Cy5-AAG GAA TTG ACG GAA GGG CA-BHQ2) were used in conjunction with the AgPath-ID One-Step RT-qPCR kit (Life Technologies, Grand Island, NY) in a 20 µL reaction. RT-qPCR reactions consisted of 1.5 µL nuclease-free water, 10 µL 2x Reaction Buffer, 1 µL 10 µM PEDVn forward and reverse primers, 1 µL 10 µM 18S forward and primers, 1 µL 10 µM 18S probe, 0.5 µL PEDV probe (10 µM), 1 µL AgPath-ID One-Step RT-PCR enzyme mix and 4 µL extracted RNA. Each RT-qPCR plate was run on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) under the following conditions: 48°C for 10 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 sec, 60°C for 40 sec. Positive and negative PCR controls were included in each run.

Statistical Analysis

Results were analyzed using the PROC GLIMMIX procedure of SAS 9.3 (SAS Institute, Inc., Cary, NC) with the fixed effects of feed matrix, treatment, and day serving as a repeated measure. Results for treatment criteria were considered significant at $P \leq 0.05$ and marginally significant from $P > 0.05$ to $P \leq 0.10$. All significant interactions remained part of the model.

Experiment 2

In the second experiment, the commercial formaldehyde product and MCFA mixture were evaluated on PEDV survival in a corn-soybean meal-based swine diet, obtained from the Kansas State University O.H. Kruse Feed Technology Innovation Center, Manhattan, KS, and spray-dried animal plasma obtained from a third-party distributor (manufactured by American Proteins, Cumming, GA). Similar to Exp. 1, the feed matrices were again first treated before inoculation with PEDV to mimic post-processing PEDV contamination. After PCR-analysis was completed, the day 3 and 21 samples were placed into a 10-d old swine bioassay to determine infectivity.

Treatment application

In order to evaluate the mitigant treatments a 4 × 2 × 7 plus 2 factorial was utilized. The four treatments; 1) positive control with PEDV and no treatment addition, 2) 0.3% commercial formaldehyde based product [Sal CURB, Kemin Industries, Des Moines, IA], 3) 1% medium chain fatty acid blend [1% MCFA; caproic, caprylic, and capric acids; 1:1:1; Sigma Aldrich, St. Louis, MO], and 4) 2% medium chain fatty acid blend [2% MCFA; caproic, caprylic, and capric acids; 1:1:1]. These treatments were applied to 2 feed matrices; 1) corn soybean meal-based swine diet and 2) spray dried animal plasma, and evaluated on 7 analysis days (d 0, 1, 3, 7, 14, 21, and 42 post inoculation). There was also 1 treatment each of PEDV negative untreated feed and plasma, which acted as controls.

In order to treat the complete feed and plasma, all treatments were added on a wt/wt basis and mixed using a lab-scale paddle mixer. The commercial formaldehyde based product and MCFA treatments were aerosolized into the mixer using an air-atomizing nozzle in order to reduce the droplet size of the liquid treatments. All treatments were mixed for a 5-minute wet mix time to ensure a uniform and complete mix.

Once the mixing was complete, a total of 22.5 g of product was collected from different locations within the mixer and added to the respective 250 mL HDPE square wide-mouth bottle, based on day and replication. In order to reduce the potential for treatment-to-treatment cross-contamination, the mixer was cleaned with soap and water between treatments. Once the treatments were added to their respective bottle, they were allowed to sit at room temperature until inoculation.

PEDV Virus isolate

The U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338) was used to inoculate feed. Virus isolation, propagation, and titration were performed in Vero cells (ATCC CCL-81) as described by Chen et al. [2]. The stock virus titer contained 4.5 x 10^6 $TCID_{50}$/ml and was diluted to 10^5 $TCID_{50}$/ml.

Inoculation

The feed was inoculated using an appropriately sized pipet to allow even distribution of the virus within the feed and plasma. For the inoculation, 2.5 mL of diluted viral inoculum was placed in each 250 mL bottle containing 22.5 grams of each feed treatment, resulting in each bottle containing a PEDV concentration of 10^4 $TCID_{50}$/g of feed. The bottles were then thoroughly shaken to ensure equal dispersion of the virus within each bottle. The samples were then stored at ambient temperature until aliquoted for viral RNA expression of PEDV at 0, 1, 3,

7, 14, 21, and 42 days post treatment via RT-qPCR. For each sample day, 100 mL of chilled PBS was placed in each 250 mL bottle containing 22.5 g of inoculated feed. Samples were then shaken to thoroughly mix and chilled at 4°C overnight. Feed matrix supernatants, including two PCR samples and a bioassay sample, were then collected and stored at -80°C until the end of the trial.

Quantitative Reverse transcriptase polymerase chain reaction (RT-qPCR)

Samples were analyzed by a duplex real-time RT-PCR (RT-qPCR) targeting the Spike gene of PEDV according to Huss et al. [21]. Briefly, RNA was isolated using a MagMAX™-96 Viral RNA Isolation Kit (Life Technologies, Grand Island, NY) [21]. Once isolated, RT-PCR was carried out in 20µL reaction volumes using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) [21].

Bioassay

The Iowa State University Institutional Animal Care and Use Committee reviewed and approved the pig bioassay protocol. A total of 60 crossbred, 10 d-old pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. Additionally, all pigs were confirmed negative for PEDV, porcine delta coronavirus (PDCoV) and transmissible gastroenteritis virus (TGEV) based on fecal swab. To further confirm PEDV-negative status, collected blood serum was analyzed for PEDV antibodies by an indirect fluorescent antibody (IFA) assay and TGEV antibodies by ELISA, both conducted at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). Pigs were allowed 2 d of adjustment to the new pens before the bioassay began. A total of 20 rooms with three pigs per room were assigned to treatment groups with 2 negative control rooms and 18 challenge rooms. The challenge rooms were represented by the day 3 and 21 samples for both feed and plasma.

Each pig within the rooms received their own dose of inoculum represented from the three aliquots collected during the RT-qPCR analysis.

During bioassays, rectal swabs were collected on d -2, 0, 2, 4, 6, and 7 days post inoculation (dpi) from all pigs and tested for PEDV RNA RT-qPCR. Following humane euthanasia at 7 dpi, small intestine, cecum, and colon samples were collected at necropsy along with an aliquot of cecal contents.

Statistical Analysis

Data of the main effects of day, treatment, feed matrix, and all associated interactions were analyzed as a completely randomized design using PROC GLIMMIX in SAS 9.4 (SAS Institute, Inc., Cary, NC). Results for treatment criteria were considered significant at $P \leq 0.05$ and marginally significant from $P > 0.05$ to $P \leq 0.10$. All significant interactions remained part of the model.

RESULTS

Experiment 1

A three-way interaction was observed in the study ($P < 0.05$). The reason a three-way interaction was observed is because over time, treatments are interacting differently within the feed matrices. Therefore, it is appropriate to evaluate the effect of each treatment over time within each feed matrix (Figure 2.1-2.4). The PEDV CT in the untreated control of the complete swine diet increased until d 21, after which it remained relative constant (Fig. 2.1). The MCFA treatment initially resulted in a decrease in the detectable genetic material at d 0 and led to the greatest overall reduction of detestable PEDV RNA in the swine diet. Each of the treatments remained relatively stable from d 21 to 42 except for essential oils. The EO treatment resulted in a further reduction of the detectable genetic material. Of the tested mitigants in the complete

swine diet, the MCFA treatment was the most effective overall, with the EO treatment reaching similar efficacy by d 42.

The PEDV Ct in the untreated control of the blood meal was similar to that of the complete swine diet, in that it increased until d 21, but was relatively similar between d 21 and d 42 (Fig. 2.2). Although the EO treatment was not effective at mitigating PEDV according to RT-qPCR through d 7, it was the most effective on d 14, 21, and 42. This response would also help to describe the treatment by day interaction within the blood meal. Interestingly, the PEDV Ct in the untreated control of the porcine meat and bone meal was highly stable throughout the experimental period, with no treatment showing substantial mitigative effects, even though differences were statistically significant (Fig. 2.3).

The PEDV Ct in the untreated control of the spray-dried porcine plasma was also relatively stable over time (Fig. 2.4). However, the commercial formaldehyde product was highly successful at mitigating PEDV according to RT-qPCR in spray-dried porcine plasma compared to other tested treatments. It is interesting to evaluate the untreated controls in each matrix over time to further emphasize that matrix is a factor affecting PEDV Ct according to RT-qPCR (Fig. 2.5). Again, the PEDV Ct in blood meal and complete swine diet increase over time consistently until d 21, but are relatively stable from d 21 to 42. Meanwhile, the porcine meat and bone meal and spray-dried porcine plasma maintain the PEDV Ct more consistently over time.

When looking at the main effects of the mitigant treatments, the commercial formaldehyde product, MCFA, EO, and OA were all effective compared to the control ($P < 0.05$). The commercial formaldehyde based product was the most effective treatment ($P < 0.05$), followed by the MCFA ($P < 0.05$). The EO and OA treatments did show some efficacy on a RT-qPCR basis and were similar to one another ($P > 0.05$). However, they were not as effective as the

commercial formaldehyde and MCFA treatments (Table 2.5, $P < 0.05$). In the feed matrices, blood meal had the least amount of detectable PEDV RNA followed by the complete swine diet, spray-dried porcine plasma, and porcine meat and bone meal, with all matrixes being different from each other (Table 2.6, $P < 0.05$). Time also affected PEDV detected by RT-qPCR, with samples analysed on d 0 and 1 being similar ($P > 0.05$), but lower than subsequent analyses (Table 2.7, $P < 0.05$). The Ct increased over time, meaning less detectable PEDV RNA, when samples were analysed on d 3, 7, 14, and 21 ($P < 0.05$).

Experiment 2

RT-qPCR Results

A three-way interaction was observed in the study ($P < 0.05$). The PEDV Ct in the untreated control of the complete diet increased in a linear fashion from d 0-42 (Figure 2.6). The treatments all had a greater decrease in detectable PEDV RNA at each analysis day than the untreated control ($P < 0.05$). In the complete swine diet, the MCFA treatments regardless of concentration were the most effective overall ($P < 0.05$). The PEDV Ct in the untreated control of the spray-dried animal plasma had the same trend for both MCFA treatments (Figure 2.7). However, the commercial formaldehyde product was highly successful at mitigating PEDV according to RT-qPCR in spray-dried animal plasma compared to the MCFA treatments. Overall, the commercial formaldehyde and both MCFA treatments reduced ($P < 0.05$) the quantity of detectible PEDV RNA compared to the control (Table 2.8). Differences were also observed between each of the feed matrixes (Table 2.9, $P < 0.0001$). Time also affected PEDV detected by RT-qPCR, with d 0 and 1 being similar ($P > 0.05$), but lower ($P < 0.05$) than d 3, 7, 14, 21, and 42 (Table 2.10). The Ct continued to increase over time with d 7 being similar to d 3

and 14 ($P > 0.05$). It was also observed that d 42 provided the lowest level of detectable PEDV RNA ($P < 0.05$).

Bioassay

In the complete feed, the only treatment that led to PEDV positive pigs was the day 0 PEDV positive feed (Table 2.11). However, in the spray-dried animal plasma, the commercial formaldehyde based product was the only treatment that led to a negative bioassay on d 3 (Table 2.12). On d 21 the commercial formaldehyde product, 1% MCFA, and PEDV positive untreated control all led to negative bioassays with the 2% MCFA treatment producing a positive bioassay 4 dpi (Table 2.12).

DISCUSSION

Surprisingly, the PEDV concentration was relatively stable in spray-dried porcine plasma and porcine meat and bone meal, while the detectable RNA reduced substantially during the initial 21-d period in the complete swine diet and blood meal. This result was also observed in Exp. 2, as the SDAP maintained a greater concentration of detectable PEDV RNA compared to the complete swine diet. Similar findings have been reported in which PEDV quantity in multiple ingredients including meat and bone meal, soy bean meal, and choline chloride have remained relatively stable over time without the presence of a liquid mitigant [5]. This was also observed in a transboundary-model study, in which the control ingredients retained a stable level of detectable PEDV RNA with a slight increase from 22.9 to 23.1 Ct over the 37-day controlled environment experiment [22]. Others have also showed that temperature, relative humidity, and the storage environment can also have an effect on PEDV RNA detectability [23]. In all, it seems that many feed ingredients can harbour PEDV genetic material up to 37 days, depending on the

type of ingredients and their storage condition. However, feed additives can reduce detection of the virus.

Under the laboratory conditions, PEDV was successfully mitigated in different feed matrices by the commercial formaldehyde product, MCFA, OA, and EO as analysed by RT-qPCR. Others have demonstrated the effectiveness of OA on PEDV mitigation, with different combinations of OA showing varying inactivation kinetics [23]. Essential oils have also been shown to be effective against RNA viruses such as dengue virus, SARS associated coronavirus, and junin virus by interfering with the virus envelope, or by masking the components that are necessary for adsorption to the host cells [24]. However, in this study the OA and EO blends were not as successful as the formaldehyde product and MCFA blend based on RT-qPCR analysis.

Formaldehyde has been shown to effectively mitigate other RNA viruses and diseases, such as classical swine fever, foot and mouth disease, and avian influenza virus [16]. Based on our study, the same results were observed compared to classical swine fever and avian influenza virus, which shows the viruses can be sensitive to the addition of aldehydes [8, 25]. However, one of the major differences observed was that an inclusion rate of 0.3% formaldehyde was used in our study compared to 1 to 2% for the avian influenza virus study [25].

The commercial formaldehyde product in this study performed similarly in complete feed to the observations made by Dee et al. [9]. What is interesting is that the formaldehyde treated meat and bone meal from Dee et al. [5] and the present study had similar results with both being PCR positive throughout the study and retaining a greater quantity of detectable RNA. This

could indicate that meat and bone meal could carry a higher risk of maintaining PEDV RNA over time.

The differences that were observed in the RT-qPCR data between the Dee et al. studies and current study could be attributed to varying commercial formaldehyde products (Termin-8 vs. Sal CURB) or application techniques. In the present study, a pilot-scale laboratory ribbon mixer fitted with aerosolizing equipment was used to mimic the commercial formaldehyde application process, and the mixer had a coefficient of mixing variation less than 7%. Alternatively, Dee et al. [9] utilized more simplistic mixing equipment that may have resulted in less efficient distribution of the product. Even though differences were observed in the RT-qPCR results for the formaldehyde treated feed matrices, the bioassay results were the same. In each instance, the formaldehyde treated feed and SDAP samples tested within the bioassay were deemed to be negative by this study and by Dee et al. [5] However, meat and bone meal was not tested in the bioassay as SDAP was thought to carry a higher risk than porcine meat and bone meal at the time of the experiment. The reason that this was thought is because of the Canadian experience around the idea that PEDV was introduced by spray dried animal plasma [26].

It has been pointed out that the presence of proteins and lipids in the viral envelope and the size of the virus, could be two factors that influence the mode of action of the mitigants [27]. This could be one of the main reasons why the virus can be sensitive to the addition of formaldehyde, which in known to cause alkylation of proteins [27]. This could also lead to the idea of other lipid solvents and medium chain fatty acids having a similar effect on the viral envelope proteins and lipids.

Medium chain fatty acids have been shown to be effective against RNA viruses such as visna virus and vesicular stomatitis virus [13]. It is thought that MCFA can cause a disruption of

the viral envelope leading to non-infection. This result has been observed by Thomar et al., using electron microscopy, in which it appears the MCFA have an influence on the viral envelope [13].

The MCFA mixture in this study performed similar to the formaldehyde treatment in complete feed but not in SDAP. However, when placed into the bioassay, both the MCFA and formaldehyde treated feed samples were deemed to be negative by RT-qPCR and clinical signs of infectivity. This, however, was not the case in the SDAP as both concentrations of the MCFA mixture caused infection from the d 3 RT-qPCR, and the 2% inclusion level at d 21 RT-qPCR samples. Because of this result, it was thought that the PEDV is interacting differently within the SDAP compared to the complete diet and taking more time for the MCFA to have an effective result. The same MCFA mixture used in this study was effective against PEDV in other feed ingredients and environmental conditions as determined by RT-qPCR and bioassay [22]. The 2% inclusion has been effective in conventional soybean meal, organic soybean meal, vitamin D, lysine, and choline chloride as determined by a swine bioassay [22]. From the RT-qPCR results and bioassay results the1% inclusion rate is as effective as the 2% in the complete diet. However, the effectiveness in SDAP seems to take more time. It is not known what the underlying reason is that MCFA are not as effective in SDAP and is why further research into potential interaction with between the virus, MCFA, and feed matrices are needed.

CONCLUSION

Experiment 1 was the first research of its kind to evaluate mitigation of post-processing PEDV contamination in swine feed and ingredients using feed based mitigants. From Exp. 1, it was determined that time, commercial formaldehyde product, MCFA, EO, and OA all enhance the RNA degradation of PEDV in the tested swine feed and ingredients, but their effectiveness varies within matrix. The detectability of the viral nucleic acid was substantially lower in blood

meal and a complete swine diet by d 21, but is relatively stable in spray-dried animal plasma and porcine meat and bone meal. From Exp. 2, it was determined that time, commercial formaldehyde based product, and MCFA enhance the RNA degradation of PEDV in swine feed and ingredients, but their effectiveness varies within matrix. Notably, the MCFA was equally as successful at mitigating PEDV as a commercially available formaldehyde product in the complete swine diet at 1% inclusion based on RT-qPCR and a 10d old swine bioassay. However, further research is needed to further explore lower levels of MCFA treatment against PEDV, evaluate the inclusion of individual MCFA's to determine if a single MCFA is leading to non-infection of PEDV, and to evaluate fat sources used in the animal feed industry that contain naturally occurring MCFA.

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TABLES AND FIGURES

Table 2.1 Proximate analysis of feed matrices (as-is basis)

Item[1]	Swine diet	Blood meal	Meat and bone meal	Spray-dried animal plasma
Moisture	11.69	9.14	3.06	9.09
Crude fat	2.61	0.53	11.10	0.00
Crude fiber	2.04	0.45	1.66	0.17
Ash	6.58	2.20	26.76	5.75
Calcium	0.96	0.12	9.34	0.08
Phosphorus	0.66	0.32	4.72	0.89

[1]W/W%= grams per 100 grams of sample;

Table 2.2 Fatty acid analysis of feed matrices (as-is basis)

Item,% [1]	Swine diet	Blood meal	Meat and bone meal	Spray-dried animal plasma
Myristic (14:0)	0.98	2.84	1.48	1.03
Myristoleic (9c-14:1)	0.00	0.20	0.25	0.37
C15:0	0.00	0.18	0.10	0.51
Palmitic (16:0)	19.67	23.57	26.01	13.63
Palmitoleic (9c-16:1)	1.42	2.23	3.35	1.07
Margaric (17:0)	0.25	0.48	0.36	0.84
10c-17:1	0.25	0.08	0.47	0.40
Stearic (18:0)	5.99	16.24	14.14	20.19
Elaidic (9t-18:1)	0.25	11.04	0.56	1.89
Oleic (9c-18:1)	31.11	19.51	40.31	9.49
Vaccenic (11c-18:1)	0.00	0.00	0.00	0.00
Linoleic (18:2n6)	34.60	7.49	5.02	34.78
Linolenic (18:3n3)	1.84	1.27	0.14	0.51
Stearidonic (18:4n3)	0.00	0.00	0.00	0.00
Arachidic (20:0)	0.27	0.72	0.25	0.41
Gonodic (20:1n9)	0.60	0.48	1.06	0.09
Homo-a-linolenic(20:3n3)	0.00	0.00	0.00	0.00
Arachidonic [20:4n6]	0.22	0.75	0.41	1.33
3n-Arachidonic (20:4n3)	0.00	0.00	0.00	0.00
EPA (20:5n3)	0.00	0.00	0.00	0.00
Behenoic (22:0)	0.20	0.77	0.19	1.38
Erucic [22:1n9]	0.00	0.00	0.00	0.00
Clupanodonic (22:5n3)	0.00	0.00	0.00	0.00
DHA (22:6n3)	0.34	0.16	0.24	0.28
Lignoceric (24:0)	0.44	0.84	0.70	0.50
Nervonic (24:1n9)	0.00	0.68	0.13	0.27

[1]W/W%= grams per 100 grams of sample;

Table 2.3 Amino acid profile of feed matrices (as-is basis)

Item[1]	Swine diet	Blood meal	Meat and bone meal	Spray-dried animal plasma
Taurine	0.01	0.01	0.10	0.01
Hydroxyproline	0.04	0.00	2.94	0.01
Aspartic Acid	2.07	7.52	4.18	8.11
Threonine	0.98	4.14	1.83	5.19
Serine	0.88	3.59	2.02	4.55
Glutamic Acid	3.44	8.02	6.63	10.54
Proline	1.15	3.84	4.67	4.03
Lanthionine	0.02	0.19	0.19	0.00
Glycine	0.85	3.40	7.40	2.75
Alanine	1.06	5.96	3.98	3.90
Cysteine	0.32	1.64	0.49	2.56
Valine	0.98	5.14	2.32	5.46
Methionine	0.42	1.04	0.79	0.94
Isoleucine	0.83	3.21	1.65	2.49
Leucine	1.84	8.76	3.42	7.69
Tyrosine	0.63	2.78	1.46	4.18
Phenylalanine	1.01	5.18	1.93	4.31
Hydroxylysine	0.04	0.05	0.35	0.00
Ornithine	0.01	0.12	0.13	0.05
Lysine	1.37	6.68	3.06	7.39
Histidine	0.59	4.36	1.17	2.58
Arginine	1.22	4.54	4.02	4.61
Tryptophan	0.25	0.57	0.35	1.51
Total	20.01	80.74	55.08	82.86

[1]W/W%= grams per 100 grams of sample;

Table 2.4 Within-day laboratory controls of PEDV-contaminated samples to evaluate the interassay variation

	Day												
Item[1]	0	1		3		7		14		21		42	
Swine diet	28.2	29.3	28.8	29.1	28.8	29.2	28.6	28.3	28.2	28.8	28.6	28.8	28.6
Blood meal	30.6	31.5	31.3	31.4	31.3	31.5	31.3	31.0	31.0	31.3	31.0	31.1	31.2
Meat and bone meal	26.4	26.2	25.9	26.2	26.2	26.0	26.1	26.0	26.0	26.3	26.2	26.3	26.2
Spray-dried animal plasma	28.2	27.0	26.6	27.3	26.6	27.7	28.1	27.4	27.2	27.3	26.5	26.8	26.7

[1]Values are represented by quantified Ct value.

Table 2.5 Main effect of mitigant treatment on detection of PEDV

Item[1]	Control	Essential oil	MCFA	Organic acids	Sodium bisulfate	Sodium chlorate	Commercial Formaldehyde[3]	SEM	*P* =
Ct value [2]	29.9[d]	30.5[c]	31.4[b]	30.4[c]	29.7[d]	29.3[e]	32.5[a]	0.08	< 0.0001

[1] A total of 582 samples were used for the analysis. A 7×4×7 factorial was utilized with 7 mitigant treatments, 4 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA utilizing RT-qPCR. Means presented are the main effect of each mitigant treatment.

[2] Cycle time required to detect the PEDV genetic material. A higher Ct value is associated with less genetic material present.

[3] Termin-8, Antiox Corp, Lawrenceville, GA

[abcde] Means within a row lacking a common superscript differ $P < 0.05$.

Table 2.6 Main effect of feed matrix on detection of PEDV

Item[1]	Swine diet	Blood meal	Porcine meat/bone meal	Spray dried animal plasma	SEM	*P* =
Ct value [2]	32.0[b]	32.9[a]	28.1[d]	29.2[c]	0.06	< 0.0001

[1] A total of 582 samples were used for the analysis. A 7×4×7 factorial was utilized with 7 mitigant treatments, 4 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA utilizing RT-qPCR. Means presented are the main effect of each feed matrix.

[2] Cycle time required to detect the PEDV genetic material. A higher Ct value is associated with less genetic material present.

Table 2.7 Main effects of sampling day on detection of PEDV

	Day								
Item[1]	0	1	3	7	14	21	42	SEM	*P* =
Ct value[2]	29.0[e]	28.8[e]	29.8[d]	30.6[c]	31.1[b]	32.1[a]	32.3[a]	0.08	< 0.0001

[1] A total of 582 samples were used for the analysis. A 7×4×7 factorial was utilized with 7 mitigant treatments, 4 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA utilizing RT-qPCR. Means presented are the main effect for each analysis day.

[2] Cycle time required to detect the PEDV genetic material. A higher Ct value is associated with less genetic material present.

[abcde] Means within a row lacking a common superscript differ, $P < 0.05$.

Table 2.8 Main effect of mitigant treatment on detection of PEDV by RT-qPCR

Item[1]	PEDV pos.	Sal CURB	1% MCFA	2% MCFA	SEM	*P* =
Ct value[2]	34.6[b]	38.3[a]	38.0[a]	38.2[a]	0.43	<0.0001

[1] A total of 168 samples were used for the analysis with each treatment represented by a mean of N=42. A 4×2×7 plus 2 factorial was utilized with 4 mitigant treatments, 2 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA utilizing RT-qPCR. Means presented are the main effect of each mitigant treatment.

[2] Cycle threshold required to detect the genetic material. A higher Ct value means less genetic material present.

[ab] Means within a row lacking a common superscript differ, ($P < 0.05$).

Table 2.9 Main effect of feed matrix on detection of PEDV by RT-qPCR

Item[1]	Feed	SDAP	SEM	*P* =
Ct value[2]	39.5	35.0	0.43	<0.0001

[1] A total of 168 samples were used for the analysis with each day represented by a mean of N=84. A 4×2×7 plus 2 factorial was utilized with 4 mitigant treatments, 2 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inoculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA. Means presented are the main effect of each feed matrix.

[2] Cycle threshold required to detect the genetic material. A higher Ct value means less genetic material present.

Table 2.10 Main effect of day post inoculation on detection of PEDV by RT-qPCR

	Day								
Item[1]	0	1	3	7	14	21	42	SEM	*P* =
Ct value[2]	33.2^{e}	34.3^{e}	35.9^{d}	36.5^{cd}	38.0^{bc}	39.0^{b}	44.0^{a}	0.13	<0.0001

[1] A total of 168 samples were used for the analysis with each day represented by a mean of N=24. A 4×2×7 plus 2 factorial was utilized with 4 mitigant treatments, 2 feed matrices, and 7 analysis days. Each feed matrix was first treated with the respective mitigant treatment and then inculated with PEDV. Sample were them analyzed over 42 days for PEDV RNA. Means presented are the main effect of each analysis day.

[2] Cycle threshold required to detect the genetic material. A higher Ct value means less genetic material present.

[ab] Means within a row lacking a common superscript differ, ($P < 0.05$)

Table 2.11 Effects of medium chain fatty acids and formaldehyde treatment of complete diet on porcine epidemic diarrhea virus (PEDV) detection from feed, pig fecal swabs and cecum contents

	PEDV N-gene Real Time-PCR, cycle threshold (Ct)						
		Fecal swabs					Cecum contents[5]
Item[1]	Feed Ct	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi
Unprocessed virus-free feed	> 45.0[3]	---[4]	---	---	---	---	> 45.0
Day 0 inoculated feed	31.0	---	---	- + -	++ -	++ -	28.0
Day 3 inoculated feed	34.1	---	---	---	---	---	> 45.0
Day 3 commercial formaldehyde[6]	37.2	---	---	---	---	---	> 45.0
Day 3 1% MCFA	42.8	---	---	---	---	---	> 45.0
Day 3 2% MCFA	42.4	---	---	---	---	---	> 45.0
Day 21 inoculated feed	37.3	---	---	---	---	---	> 45.0
Day 21 commercial formaldehyde[6]	40.4	---	---	---	---	---	> 45.0
Day 21 1% MCFA	> 45.0	---	---	---	---	---	> 45.0
Day 21 2% MCFA	> 45.0	---	---	---	---	---	> 45.0

[1]An initial tissue culture containing 10^6 $TCID_{50}$/mL PEDV was diluted to 10^5 $TCID_{50}$/mL PEDV. Each treatment was inoculated with the 10^5 $TCID_{50}$/mL PEDV resulting in 10^4 $TCID_{50}$/g PEDV inoculated feed matrix. Three feed samples per day and treatment were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. Treatment supernatant collected on d 0, 3, and 21 were utilized for the bioassay and were selected based on the RT-qPCR results. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per treatment (10 mL per pig). Pigs were inoculated at d 12 of age.

[2]Day post inoculation.

[3]A cycle threshold (Ct) of >45 was considered negative for presence of PEDV RNA. Feed Ct values were analyzed by RT-qPCR at Kansas State University.

[4] In each instance a (–) signals a negative pig in the bioassay and a (+) represents a positive in the bioassay. Each day post inoculation within each treatment has three symbols with each row and column, which represents one of the three pigs in each treatment.

[5] Each cecum content value represents the mean of 3 pigs per treatment and was analyzed by RT-qPCR at Iowa State University.

[6] Sal CURB, Kemin Industries, Des Moines, IA

Table 2.12 Effects of medium chain fatty acids and formaldehyde treatment of spray dried porcine plasma on porcine epidemic diarrhea virus (PEDV) detection from plasma, pig fecal swabs and cecum contents

	PEDV N-gene Real Time-PCR, cycle threshold (Ct)						
		Fecal swabs					Cecum contents
Item[1]	Plasma Ct	0 dpi[2]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi
Unprocessed virus-free feed	> 45.0[3]	---[4]	---	---	---	---	> 45.0
Day 0 inoculated plasma	30.1	---	+++	+++	+++	+++	27.8
Day 3 inoculated plasma	31.6	---	+++	+++	+++	+++	29.8
Day 3 commercial formaldehyde[6]	34.5	---	---	---	---	---	> 45.0
Day 3 1% MCFA	34.0	–	+++	+++	+++	+++	30.4
Day 3 2% MCFA	31.1	–	+++	+++	+++	+++	29.4
Day 21 inoculated plasma	36.0	---	---	---	---	---	> 45.0
Day 21 commercial formaldehyde[6]	> 45.0	---	---	---	---	---	> 45.0
Day 21 1% MCFA	31.7	---	---	---	---	---	> 45.0
Day 21 2% MCFA	31.5	---	---	- + -	+++	+++	31.3

[1]An initial tissue culture containing 10^6 $TCID_{50}$/mL PEDV was diluted to 10^5 $TCID_{50}$/mL PEDV. Each treatment was inoculated with the 10^5 $TCID_{50}$/mL PEDV resulting in 10^4 $TCID_{50}$/g PEDV inoculated feed matrix. Three feed samples per day and treatment were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. Treatment supernatant collected on d 0, 3, and 21 were utilized for the bioassay and were selected based on the RT-qPCR results. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per treatment (10 mL per pig). Pigs were inoculated at d 12 of age.

[2]Day post inoculation.

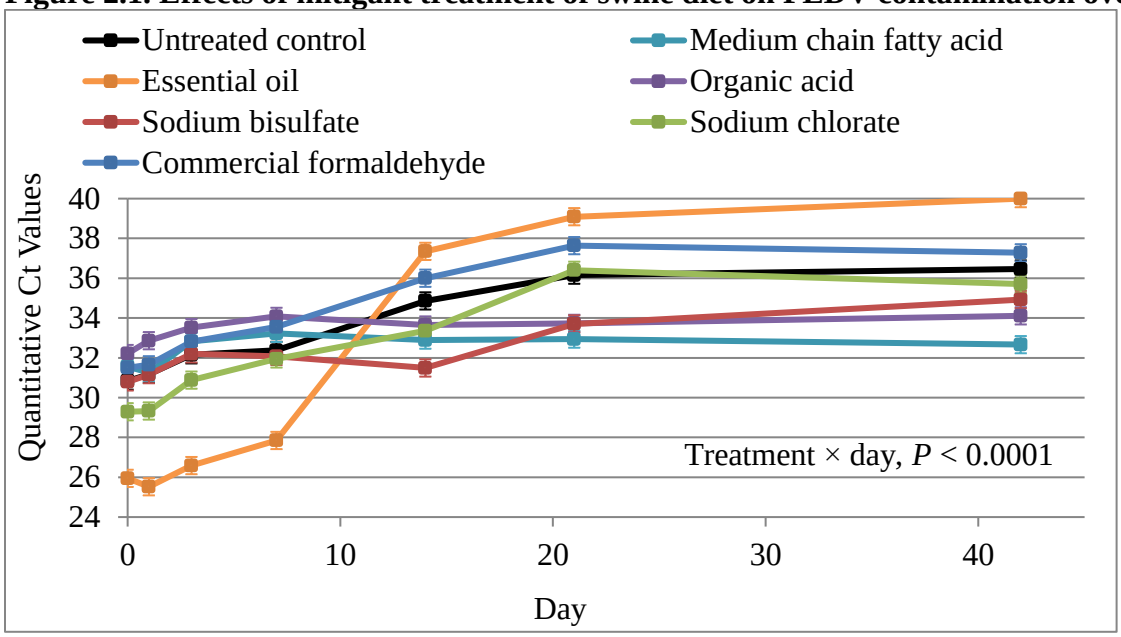
[3]A cycle threshold (Ct) of >45 was considered negative for presence of PEDV RNA. Feed Ct values were analyzed by RT-qPCR at Kansas State University.

[4] In each instance a (–) signals a negative pig in the bioassay and a (+) represents a positive in the bioassay. Each day post inoculation within each treatment has three symbols with each row and column, which represents one of the three pigs in each treatment.

[5] Each cecum content value represents the mean of 3 pigs per treatment and was analyzed by RT-qPCR at Iowa State University.

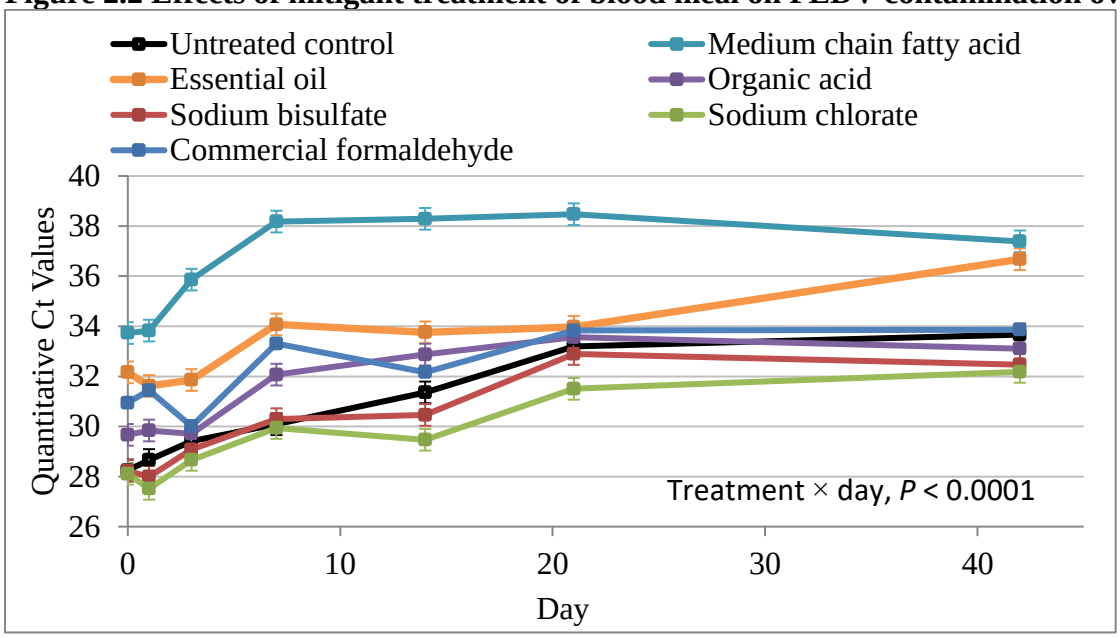
[6] Sal CURB, Kemin Industries, Des Moines, IA

Figure 2.1. Effects of mitigant treatment of swine diet on PEDV contamination over time.



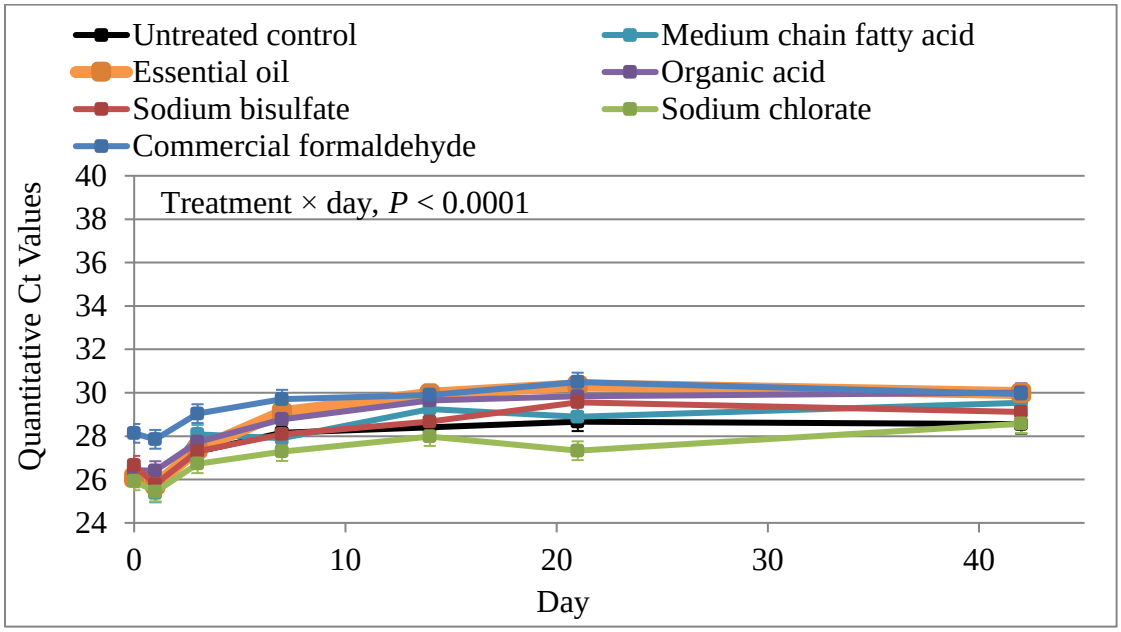
Data demonstrates the effects of mitigant treatments to prevent or mitigate PEDV post-processing contamination over time in a phase 3 complete swine nursery diet. Quantitative Ct values of PEDV represent contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Figure 2.2 Effects of mitigant treatment of blood meal on PEDV contamination over time.



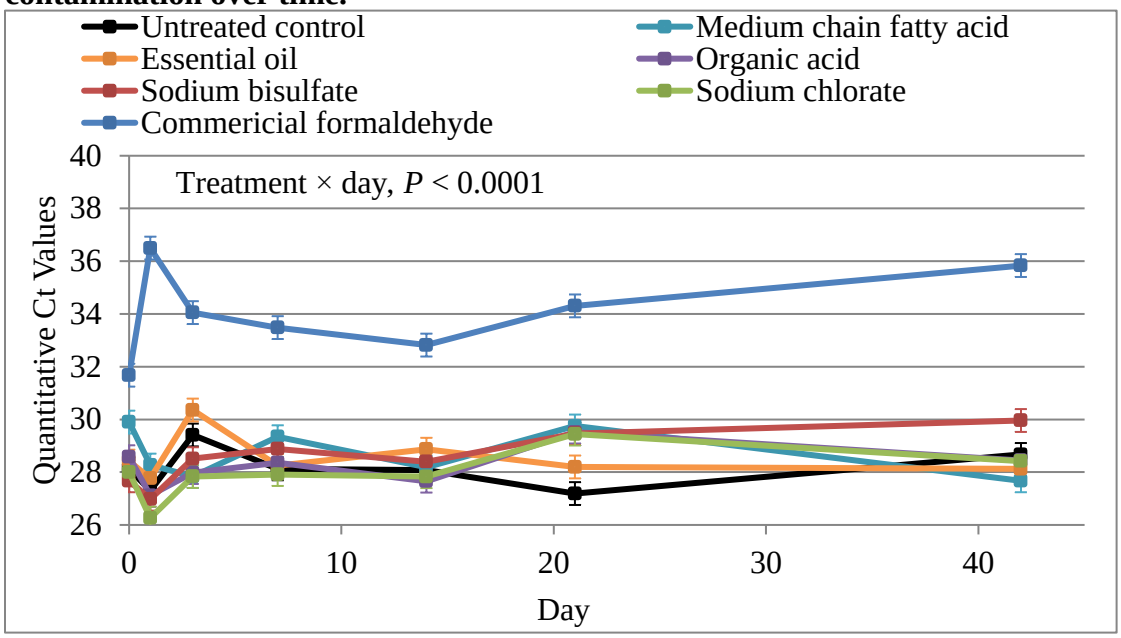
Data demonstrates the effects of mitgant treatments to prevent or mitigate PEDV post-processing contamination over time in avian blood meal. Quantitative Ct values of PEDV represents contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Figure 2.3 Effects of mitigant treatment of meat and bone meal on PEDV contamination over time.



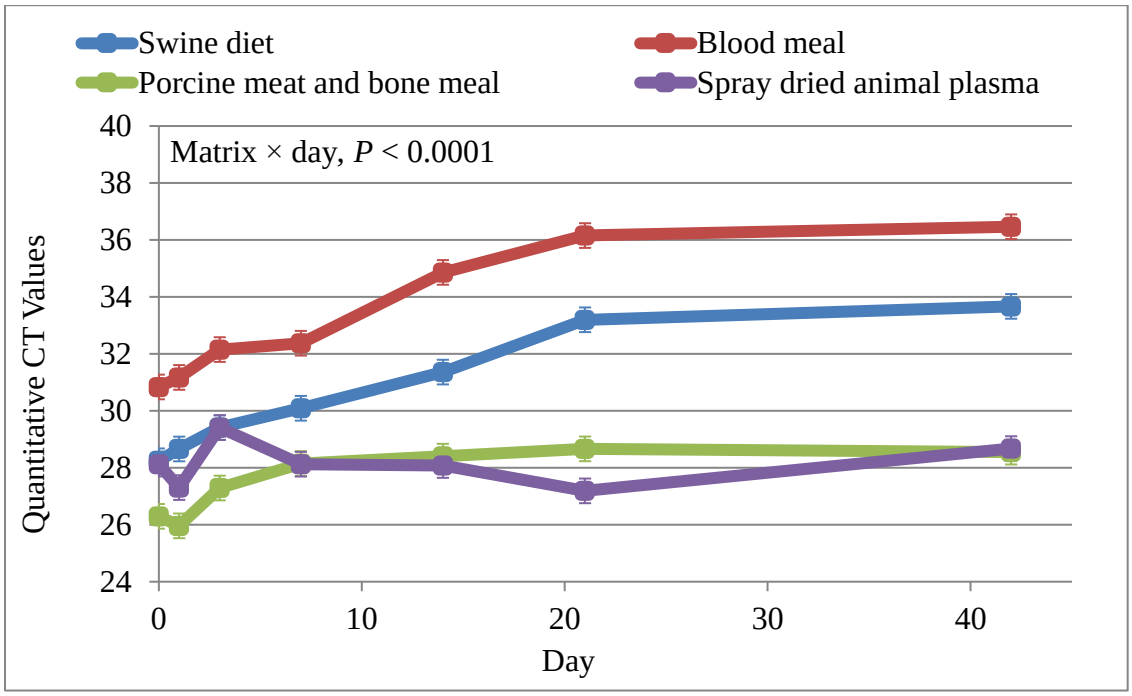
Data demonstrates the effects of mitigant treatments to prevent or mitigate PEDV post-processing contamination over time in porcine meat and bone meal. Quantitative Ct values of PEDV represent contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Figure 2.4 Effects of mitigant treatment of spray-dried animal plasma on PEDV contamination over time.



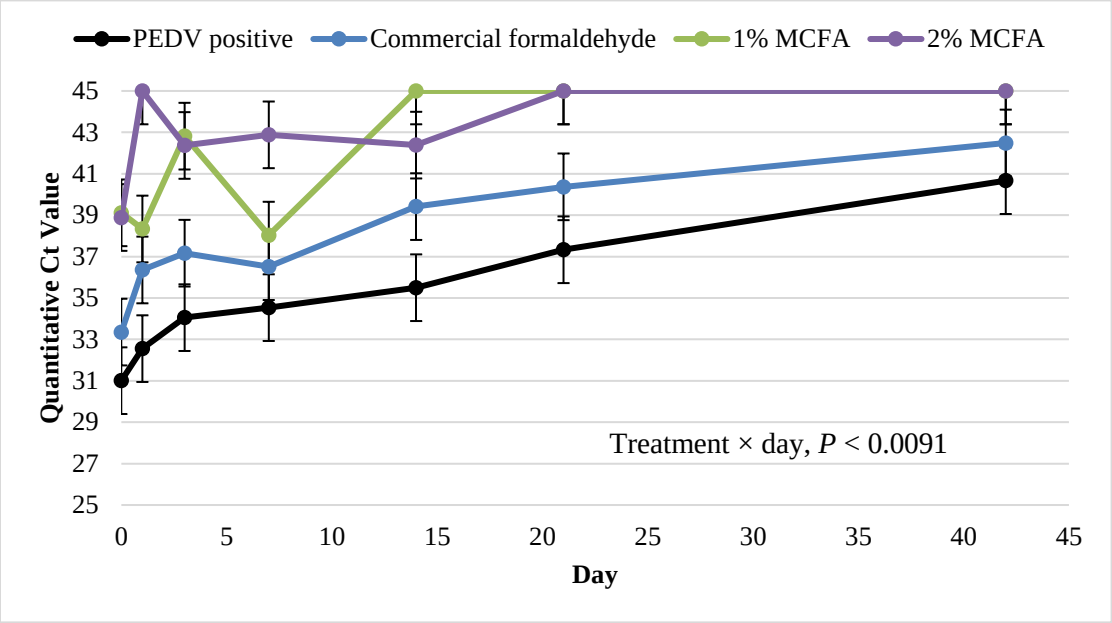
Data demonstrates the effects of various mitigant treatments to prevent or mitigate PEDV post-processing contamination over time in spray-dried animal plasma. Quantitative Ct values of PEDV represent contamination.

Figure 2.5 Effects of feed matrix on PEDV contamination over time.



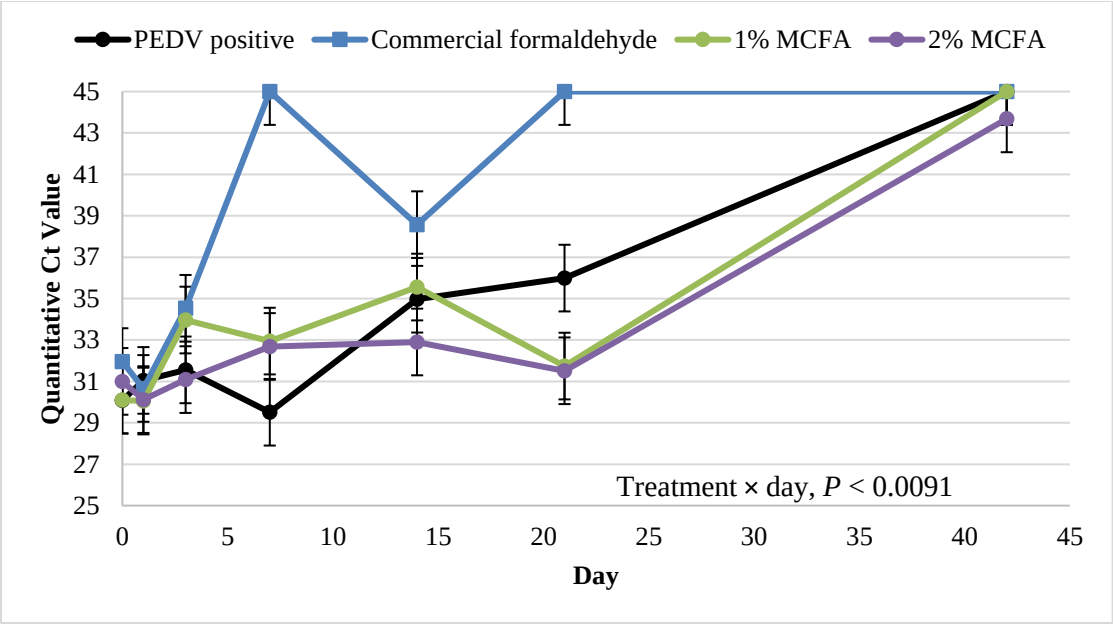
Data demonstrates the effects of feed matrix without treatment (untreated controls of Figures 1-4) to prevent or mitigate PEDV post-processing contamination over time. Quantitative Ct values of PEDV represent contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Figure 2.6 Influence of mitigant treatment on RT-qPCR detection of PEDV in complete swine diet.



Data demonstrates the effects of various treatments to prevent or mitigate PEDV post-processing contamination over time in a phase 3 complete swine nursery diet. Quantitative Ct values of PEDV represent contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Figure 2.7 Influence of mitigant treatment on RT-qPCR detection of PEDV in post-treatment PEDV-inoculated spray dried animal plasma stored at room temperature.



Data demonstrates the effects of various treatments to prevent or mitigate PEDV post-processing contamination over time in sprayed-dried animal plasma. Quantitative Ct values of PEDV represent contamination as measured by RT-qPCR. The lower the Ct value, the greater quantity of PEDV RNA genetic material detected.

Chapter 3 - Assessing the effects of medium chain fatty acids and fat sources on PEDV infectivity

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ABSTRACT

Research has confirmed that chemical treatments, such as medium chain fatty acids (MCFA) and commercial based formaldehyde-based products, can reduce the risk of porcine epidemic diarrhea virus (PEDV) cross-contamination in feed. However, the efficacy of individual MCFA levels are unknown. The overall objective of this study was to compare the efficacy of commercially-available sources of MCFA and other fat sources versus a synthetic custom blend of MCFA to minimize the risk of PEDV cross-contamination as measured by qRT-PCR and bioassay. Treatments were arranged in a 17 × 4 plus 1 factorial with 17 chemical treatments inoculated with PEDV post application: 1) Positive control with PEDV and no chemical treatment, 2) 0.325% commercially-available formaldehyde-based product; 3) 1% blend of 1:1:1 caproic, caprylic, and capric acids and applied with an aerosolizing nozzle; 4) treatment 3 applied directly into the mixer without an aerosolizing nozzle; 5) 0.66% caproic acid; 6) 0.66% caprylic acid; 7) 0.66% capric acid; 8) 0.66% lauric acid; 9) 1% blend of 1:1 capric and lauric acids; 10) 0.3% commercially-available dry C12 product; 11) 1% canola oil; 12) 1% choice white grease; 13) 2% coconut oil; 14) 1% coconut oil; 15) 2% palm kernel oil; 16) 1% palm kernel oil; 17) 1% soy oil; and; 4 analysis days (0, 1, 3, and 7 post inoculation); and 1 treatment of PEDV-negative feed without chemical treatment. Feed was first treated, then inoculated with PEDV and stored at room temperature until analyzed by qRT-PCR. The analyzed values represent cycle threshold (Ct). There was a treatment × day interaction ($P < 0.002$) for detectable PEDV RNA. The magnitude of the increase in Ct value from d 0 to 7 was dependent upon the individual treatments. Feed treated with individual MCFA, 1% MCFA blend, or commercial based formaldehyde had fewer ($P < 0.05$) detectable viral particles than all other treatments. Commercial-based formaldehyde, 1% MCFA, 0.66% caproic, 0.66% caprylic,

and 0.66% capric acids all led to negative clinical signs and qRT-PCR results when the treatments were placed into a 10-d old pig bioassay. In summary, MCFA were equally as successful at mitigating PEDV as a commercially based formaldehyde product in a complete swine diet at 1% inclusion and as individual fatty acids.

Key words: Porcine Epidemic Diarrhea Virus (PEDV), medium chain fatty acids, fat source, swine

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is known to be spread primarily by fecal-oral contamination, but controlled and epidemiological research has shown PEDV can be spread by complete feed and ingredients (Dee et al., 2014; Pasick et al., 2014; Schumacher et al., 2016). Fecal contamination in the feed supply chain may enter with ingredients or from cross-contamination during the manufacturing, transportation, and storage of feed (Cochrane et al., 2016a). Thermal processing helps reduce the risk of this transmission, but is a point-in-time mitigant that offers no residual protection from post-processing cross-contamination (Cochrane et al., 2017). Biosecurity can limit this cross-contamination, but is challenging to implement across the feed manufacturing and delivery industry (Cochrane et al., 2016a).

Research has demonstrated mitigation additives can reduce the likelihood of viral contamination in a feed matrix. Additionally, mitigation additives can provide residual activity (Cochrane et al., 2016b; Dee et al., 2016 Trudeau et al., 2016). For example, formaldehyde-based products are highly effective against PEDV. However, they are not labeled for its control (Food and Drug Administration, 21 CFR § 573.460, 2017), and can be perceived negatively by consumers (Jones 2011). An alternative mitigation additive is medium chain fatty acids (MCFA).

Initial research has demonstrated that a 2% and 1% inclusion of a 1:1:1 ratio of caproic, caprylic, and capric acids is as effective as formaldehyde-based products at reducing the quantity and infectivity of PEDV RNA in a complete swine diet (Cochrane et al., 2015, Cochrane et al., 2016b). Currently it is unknow if the specific ratio of MCFA needs to be utilized to reduce PEDV. Furthermore, it is not known if a specific fatty acid is driving the response to the MCFA blend, or if it is simply an effect of added fat in the diet. The MCFA blend has also been applied using a special aerosolized nozzle to reduce droplet size which is a similar process used for application of commercial formaldehyde-based products (Cochrane et al., 2015, Cochrane et al., 2016b). This would require special equipment to be installed into feed mills thus making it more difficult to utilize MCFA in complete swine feed. It is unknown if direct addition into the mixer would be sufficient for MCFA application making the use of MCFA a more realistic approach.

Fat sources used in the swine industry also contain MCFA and medium chain triglycerides (MCT). Common fats used in the United States swine industry include choice white grease and soy oil which contain low levels of MCT and MCFA. However, other countries utilize fat sources such as coconut oil and palm kernel oil which contain higher levels of MCT and MCFA. However, it is not known how effective the products would be as a PEDV reduction strategy because of the MCFA being contained within the MCT molecules.

Therefore, the objectives of this experiment were to evaluate if: 1) a specialized aerosolized nozzle is necessary to apply MCFA for maximum PEDV mitigation; 2) the 1% blend of MCFA has greater efficacy than each of the fatty acids alone or compared to commercial MCFA products; and 3) the 1% MCFA blend has greater efficacy than a 1% inclusion of other fat sources.

MATERIALS AND METHODS

The Iowa State University Institutional Animal Care and Use Committee approved the pig bioassay protocol. In order to evaluate the use of chemical treatments and fat sources on PEDV survival, a corn and soybean meal-based swine diet was manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center in Manhattan (Table 3.1).

Treatment application

The diet was either left untreated (control) or was mixed with different mitigants prior to inoculation with PEDV to test residual mitigation capability. The 18 treatments were 1) positive control without mitgant treatment but inoculated with PEDV; 2) 0.325% commercial based formaldehyde product (Kemin Industries, Des Moines, IA; Sal CURB); 3) 1% blend of 1:1:1 caproic, caprylic, and capric acids and applied with an aerosolizing nozzle (MCFA aerosolized); 4) treatment 3 applied directly into the mixer without an aerosolizing nozzle (MCFA non-aerosolized); 5) 0.66% caproic acid; 6) 0.66% caprylic acid; 7) 0.66% capric acid; 8) 0.66% lauric acid; 9) 1% blend of 1:1 capric and lauric acids; 10) 0.3% commercially-available dry C12 product (FRA C12, Framelco, Raamsdonksveer, Netherlands;); 11) 1% canola oil; 12) 1% choice white grease; 13) 2% coconut oil; 14) 1% coconut oil; 15) 2% palm kernel oil; 16) 1% palm kernel oil;17) 1% soy oil; and 1 treatment of PEDV-negative feed without mitigant treatment. Treatments 5-8 were included at rates derived from the original 2% inclusion of the 1:1:1 ratio of caproic, caprylic, and capric acids utilized from Cochrane et al., (2015). Thus a 1:1:1 ratio of three products included to 2% would be equal to 0.66% of each product. Choice white grease, soy oil, canola oil, palm kernel oil, and coconut oil were analyzed for their fatty acid profiles at the Agricultural Experiment Station Chemical Laboratories, University of Missouri-Columbia, College of Agriculture, Food and Natural Resources using AOAC method 996.06.

All treatments were added on a weight to weight basis and mixed using a laboratory-scale paddle mixer (Cabela's Inc., Sidney, NE) that had been validated for mixing efficiency. Mixers were sanitized between treatments. The commercially based formaldehyde and MCFA aerosolized treatment were mixed using an air atomizing nozzle to reduce the droplet size of the liquid treatments. The remaining treatments were added directly to the mixer. After each treatment was mixed, 22.5 g of feed was collected from 8 different locations within the mixer to create a composite subsample and then added to 1 of 12-250 mL high-density polyethylene, square, wide-mouth bottles (Thermo Fisher Scientific, Waltham, MA) per treatment (3 replications per treatment × 4 analysis days = 12 bottles per treatment for inoculation).

Inoculation

The U.S. PEDV prototype strain cell culture isolate USA/IN/2013/19338, passage 8 (PEDV19338) was isolated, propagated, and titrated in Vero cells (ATCC CCL-81) as described by Chen et al. (2014). The stock virus titer contained 4.5 x 10^6 $TCID_{50}$/ml, and was diluted to 10^5 $TCID_{50}$/mL using cell culture media to create the viral inoculum. In all bottles except the negative controls, 2.5 mL of inoculum was added to the 22.5 grams of each feed treatment. Each bottle was thoroughly shaken to ensure equal dispersion of the virus, resulting in each inoculated bottle containing feed with a PEDV concentration of 10^4 $TCID_{50}$/g.

RT-qPCR Analysis

Samples were either aliquoted for viral RNA expression of PEDV via qRT-PCR immediately (d 0) or stored at room temperature for analysis on d 1, 3, and 7 post inoculation. For analysis, 100 mL of chilled PBS was added to each bottle, which were then shaken and chilled overnight at 4°C. Three samples of supernatant were then collected, including two PCR samples and a bioassay sample, and stored at -80°C until the end of the experiment. Samples were analyzed by

a duplex real-time RT-PCR (RT-qPCR) targeting the Spike gene of PEDV according to Huss et al. (2017). Results are reported in cycle threshold (Ct). A higher Ct value means less genetic material was present.

Bioassay Analysis

Fifteen treatments were selected for the bioassay and were the d 0 post-inoculation: 1) negative control with no PEDV and no mitigant treatment; or 2) positive control with PEDV and no chemical treatment; or from d 1 post-inoculation: 3) positive control with PEDV and no chemical treatment; 4) 0.325% commercial based formaldehyde; 5) 1% MCFA non-aerosolized; 6) 0.66% caproic acid; 7) 0.66% caprylic acid; 8) 0.66% capric acid; 9) 0.66% lauric acid; 10) 0.3% FRA C12; 11) 1% canola oil; 12) 1% choice white grease; 12) 1% coconut oil; 14) 1% palm kernel oil; 15) 1% soy oil.

A total of 45 crossbred, 10 d-old pigs of mixed sex were sourced from a single commercial, crossbred farrow-to-wean herd with no prior exposure to PEDV. The Iowa State University Veterinary Diagnostic Laboratory confirmed all pigs negative for PEDV, porcine delta coronavirus, and transmissible gastroenteritis virus by fecal swab. Blood serum analysis further confirmed no prior PEDV exposure through analysis of PEDV antibodies by an indirect fluorescent antibody assay. Pigs were allowed 2 d of adjustment prior to beginning the bioassay. Forty-five pigs were assigned to one of 15 rooms (3 pigs per room). Each room was represented by one of the 15 treatments mentioned above. Each pig was given the respected treatment inoculum via oral gavage according to methods reported by Thomas et al. (2015). Rectal swabs were collected on d -2, 0, 2, 4, 6, and 7 post inoculation (dpi) from all pigs and tested for PEDV RNA by qRT-PCR. Following humane euthanisia at 7 dpi, small intestine, cecum, and colon samples were collected at necropsy along with an aliquot of cecal contents as described by

Schumacher et al., 2017. A negative bioassay was determined if all rectal swabs and cecum contents had non-detectable levels of PEDV. If any samples had detectable RNA, the result was considered a positive bioassay.

Statistical analysis

Data of the main effects of treatment, day, and the interaction were analyzed as a completely randomized design using PROC GLIMMIX in SAS v9.4 (SAS Institute, Inc., Cary, NC). Results were considered significant if $P \leq 0.05$ and marginally significant if $0.05 < P \leq 0.10$. The PEDV negative control with no PEDV and no mitigation treatment was not included in the statistical analysis as the samples were only analysed on d 0 to show that no PEDV RNA was detected in the complete feed.

RESULTS

Fatty acid analysis

Fatty acid profiles for choice white grease, soy oil, canola oil, palm kernel oil, and coconut oil are displayed in Table 3.2. Coconut oil and palm kernel oil provided the greatest concentration of MCFA.

qRT-PCR Results

No PEDV RNA was detected in the feed sample without PEDV or mitigation treatment. There was a treatment × day interaction (P =0.0002) for detectable PEDV RNA (Table 3.3). The MCFA treatments of 1% MCFA (aerosolized and not aerosolized), 0.66% caproic, and 0.66% caprylic each differed ($P < 0.05$) from the commercial formaldehyde treatment on d 0 showing a greater magnitude of initial reduction of detectable PEDV RNA. However, by d 7, 0.66% caproic, and 0.66% caprylic were similar ($P > 0.05$) to the commercial formaldehyde demonstrating that after d 0, the magnitude of decrease of the detectable PEDV RNA was greater

in the commercial formaldehyde product. This goes to show that the magnitude of the increase in Ct value on the initial analysis day and from d 0 to 7 was dependent upon the individual treatments. For example, an 8.7 increase in Ct was noted in the commercial based formaldehyde product compared to a 3.7 Ct increase in choice white grease by d 7.

As time increased, each analysis day had less ($P < 0.0001$) detectable PEDV RNA compared to each previous analysis day (Table 3.4). Mitigation treatment also impacted ($P < 0.0001$) the quantity of detectable PEDV RNA (Table 3.4). The MCFA blends (1% MCFA and 1% capric:lauric), caproic acid, caprylic acid, capric acid, lauric acid, and commercial based formaldehyde reduced ($P < 0.05$) the quantity of detectable PEDV RNA compared to the positive control. There was no evidence delivery method impacted ($P > 0.05$) Ct value of the 1% MCFA blend. Also, there was no evidence the feed with FRA C12, choice white grease, soy oil, canola oil, palm kernel oil, and coconut oil, regardless of inclusion level had a different ($P > 0.05$) Ct value compared to the PEDV positive control feed.

Bioassay Results

There was no evidence of infection in pigs challenged with the PEDV-negative feed. However, pigs receiving the PEDV-infected feed without chemical mitigation had evidence of PEDV infectivity. Treatments without detectable evidence of PEDV infection were feed treated with commercial-based formaldehyde, MCFA blend, caproic acid, caprylic acid, and capric acid (Table 3.5). Pigs challenged with feed containing lauric acid, FRA C12, choice white grease, or any of the vegetable oil sources had evidence of PEDV infectivity. Notably, pigs receiving the coconut oil treatment had no detectable PEDV RNA until d 7.

DISCUSSION

Previous research demonstrating the efficacy of MCFA as a PEDV mitigant had applied commercial based formaldehyde products and MCFA using an aerosolizing nozzle (Cochrane et al., 2015; Cochrane et al., 2016b). This system reduces droplet size to increase the surface area of the applied liquids. These systems are expensive to install and require greater maintenance than typical fat application systems. This research demonstrates that an aerosolizing liquid application system is not necessary for MCFA to have maximum efficacy for PEDV mitigation, which is an important consideration for feed mills.

In agreement with previous research, MCFA and formaldehyde-based products reduced the detectable PEDV RNA by qRT-PCR in swine feed (Cochrane et al., 2016b; Dee et al., 2016; Gebhardt et al. 2017). In general, the 1% MCFA blend performed similarly to previous research by Cochrane et al., (2016b). However, this experiment goes further than previous research to identify the most effective components of this MCFA blend. The mitigation success of the 1% MCFA blend is driven by caprylic acid and caproic acid, as they provided the greatest reduction in detectable PEDV (35.5 and 36.3 Ct, respectively) compared to capric acid (32.4 Ct). While, the 0.66% inclusion of any of the three led to a negative bioassay, none had similar reduction in detectable PEDV of the 1% MCFA blend (37.4 Ct). This suggests there is a possible synergistic effect of the MCFA when in combination with one another, but additional research is necessary to identify the minimum inhibitory concentration of MCFA alone or in combination in various feed matrices.

Medium chain fatty acids have been shown to destabilize the cellular membrane bi-layer of bacteria by incorporating themselves into the lipid bi-layer because of the similar hydrophilic/lipophilic balance (Desbois and Smith, 2010; Kim and Rhee, 2013). This in turn

causes pores to be created altering the cellular membrane and leading to cell death (Desbois and Smith, 2010; Kim and Rhee, 2013). Because of this mode of action with bacteria, we hypothesize that the greater efficacy of the blend and shorter MCFA may be due to how these specific fatty acids interact with the lipid and protein components of the envelope of the virus. Specifically, we believe that the relatively neutral pH of MCFA allow these fatty acids to interact with the lipids within the viral envelope. This would then cause pores to be created and lead to destabilization of the viral envelope with an effect similar to the bacterial mode of action. If this mode of action is true, then the viral envelop would not be able to attach to the host and lead to no replication.

Research in other enveloped viruses has demonstrated the success of MCFA as a mitigant (Thormar et al., 1987; Hilmarsson et al., 2006), but this mode of action needs to be confirmed in PEDV. Thormar et al., (1987) suggested that the MCFA are disrupting, and depending upon the MCFA and concentration, disintegrating the viral envelopes. Our hypothesis would then agree with this statement, and potentially describe why the level of detectable genetic material decreases over time at a faster rate when MCFA is included in the diet.

As our working hypothesis is that the MCFA can approach the PEDV envelope and cause destabilization, it was necessary to confirm that this effect is unique to MCFA, and not to other lipids. The natural triglycerides (choice white grease coconut oil, corn oil, palm kernel oil, and soy oil) provided no benefit to reduce viral RNA, and feeds treated with all resulted in infectivity. However, pigs receiving the coconut oil treatment had delayed clinical signs of PEDV. Based on its fatty acid analysis, coconut oil had the greatest MCFA concentrations of the natural fat sources. The total quantity of caproic, caprylic, and capric acids in the final diet were 0.11% or 0.21 in the 1% or 2% coconut oil treatments, respectively. While this is lower than

0.66% of each individual fatty acid or the 1% MCFA blend that demonstrated no infectivity, the small quantity of MCFA in the coconut oil may have led to a delay in PEDV infectivity. Another natural fat source with known levels of MCFA is palm kernel oil, but the concentration of caproic, caprylic, and capric acids were lower than in coconut oil. Treatments with palm kernel oil had no protection from PEDV. Furthermore, the MCFA in either the coconut or palm kernel oil treatments were presumably medium chain triglycerides. It is thought that longer MCFA, such as lauric and myristic acids, are too lipophilic to approach the PEDV cellular membrane, while those bound in a triglyceride need hydrolysis from lipase to carry on its mode of action.

In summary, this research suggests a specialized aerosolized nozzle is not necessary to apply MCFA for PEDV mitigation. It was also demonstrated that 0.66% caproic, 0.66% caprylic, and 0.66% capric acids have similar efficacy as a commercial based formaldehyde or the 1% MCFA blend when placed into a 10 d old swine bioassay. It was also shown that these medium chain fatty acids are more effective at mitigating PEDV than longer chain fatty acids or triglycerides. However, further research needs to be carried out to evaluate alternative MCFA combinations, and MCFA concertation levels against PEDV.

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TABLES AND FIGURES

Table 3.1 Diet composition

Item	Negative control
Ingredient, %	
Corn	79.30
Soybean meal, 46.5% CP	15.70
Choice white grease	1.00
Monocalcium phosphate	1.40
Limestone, ground	1.15
Salt	0.50
L-Threonine	0.03
Trace mineral premix[1]	0.15
Sow add pack[2]	0.50
Vitamin premix[3]	0.25
Phytase[4]	0.02
Total	100.00
Formulated analysis, %	
Dry matter	91.4
Crude protein	17.1
Crude fiber	3.7
Ether extract	3.5
Ca	0.78
P	0.52

[1]Each kilogram contains 26.4 g Mn, 110 g Fe, 110 g Zn, 11g Cu, 198 mg I, and 198 mg Se.
[2]Each kilogram contains 220,000 mg choline, 88 mg biotin, 660 mg folic acid, 1,980 mg pyridoxine.
[3]Each kilogram contains 4,400,000 IU vitamin A, 660,000 IU vitamin D_3, 17,600 IU vitamin E, 1,760 mg menadione, 3,300 mg riboflavin, 11,000 mg pantothenic acid, 19,800 mg niacin, 15.4 mg vitamin B_{12}.
[4]High Phos 2700 GT, DSM Nutritional Products, Parsippany, NJ.

Table 3.2 Fatty acid profile for each fat source

Item[1]	Soy oil	Coconut oil	Canola oil	Choice white grease	Palm kernel oil
Caproic C6:0	0.0	0.3	< 0.1	< 0.1	0.1
Caprylic C8:0	0.0	5.1	0.0	< 0.1	2.5
Capric C10:0	0.0	5.2	< 0.1	0.1	2.9
Lauric C12:0	0.0	46.8	< 0.1	0.1	45.8
Myristic (14:0)	0.1	19.5	0.1	1.4	16.6
Myristoleic (9c-14:1)	0.0	0.0	0.0	< 0.1	0.0
C15:0	< 0.1	< 0.1	< 0.1	0.1	< 0.1
Palmitic (16:0)	10.8	10.2	3.9	22.4	9.2
Palmitoleic (9c-16:1)	0.1	< 0.1	0.2	2.5	< 0.1
Margaric (17:0)	0.1	< 0.1	0.1	0.4	< 0.1
10c-17:1	0.0	0.0	0.0	0.0	0.0
Stearic (18:0)	3.9	3.3	1.7	9.7	2.4
Elaidic (9t-18:1)	0.0	< 0.1	< 0.1	0.0	< 0.1
Oleic (9c-18:1)	20.2	7.3	59.1	40.9	17.2
Vaccenic (11c-18:1)	1.5	0.2	3.0	2.8	0.0
Linoleic (18:2n6)	53.6	1.8	19.0	14.2	2.8
Linolenic (18:3n3)	7.9	< 0.1	8.8	0.5	< 0.1
Stearidonic (18:4n3)	0.0	0.0	0.0	0.0	0.0
Arachidic (20:0)	0.3	0.1	0.6	0.2	0.1
Gonodic (20:1n9)	0.2	0.1	1.6	0.9	0.11
C20:2	0.1	0.0	0.1	0.7	< 0.1
Homo-a-linolenic(20:3n3)	0.0	0.0	0.0	0.0	< 0.1
Arachidonic [20:4n6]	0.0	0.0	0.0	0.3	< 0.1
3n-Arachidonic (20:4n3)	0.0	0.0	0.0	0.0	< 0.1
EPA (20:5n3)	0.0	0.0	0.0	< 0.1	< 0.1
Behenoic (22:0)	0.4	< 0.1	0.3	< 0.1	< 0.1
Erucic [22:1n9]	< 0.1	0.0	< 0.1	< 0.1	< 0.1
Clupanodonic (22:5n3)	0.0	0.0	0.0	< 0.1	< 0.1
DHA (22:6n3)	0.0	0.0	0.0	< 0.1	< 0.1
Lignoceric (24:0)	0.1	< 0.1	0.2	< 0.1	0.1
Nervonic (24:1n9)	0.0	0.0	0.2	< 0.1	0.0

[1]Expressed as percent of total fat. W/W%= grams per 100 grams of sample. Results are expressed on an "as is" basis unless otherwise indicated.

Table 3.3 Effect of treatment × day post inoculation on PEDV detection using RT-PCR

	Day					Treatment × day
Item[1,2]	0	1	3	7	SEM	*P* =
PEDV positive	28.3^{wvxyz}	29.7^{rstuv}	31.3^{nopq}	32.7^{klmn}	0.5239	0.0002
0.325% Commercial formaldehyde	28.7^{uvwxy}	33.0^{jklm}	35.0^{fgh}	37.3^{cd}		
1% MCFA (aerosolized)[4]	33.3^{ijkl}	36.3^{def}	38.3^{bc}	39.0^{ab}		
1% MCFA (non-aerosolized)[5]	34.3^{ghij}	38.3^{bc}	37.0^{cde}	40.0^{a}		
0.66 % Caproic acid	33.7^{hijk}	35.0^{fgh}	36.3^{def}	37.0^{cde}		
0.66% Caprylic acid	34.3^{ghij}	35.7^{efg}	38.0^{bc}	37.3^{cd}		
0.66% Capric acid	29.3^{stuvw}	30.7^{opqrs}	34.0^{ghij}	35.3^{fg}		
0.66 % Lauric acid	28.3^{vwxyz}	30.7^{opqrs}	32.7^{klmn}	34.7^{ghi}		
1% Capric:Lauric acids[6]	29.0^{tuvwx}	31.7^{mnop}	34.3^{ghij}	34.3^{ghij}		
0.3% FRA C12[7]	28.0^{wxyz}	30.7^{opqrs}	31.7^{mnop}	33.7^{hijk}		
1% Canola oil	27.0^{z}	30.7^{opqrs}	31.0^{opqr}	31.7^{mnop}		
1% Choice white grease	28.3^{vwxyz}	30.0^{qrstu}	30.7^{opqrs}	32.0^{lmno}		
1% Coconut oil	28.0^{wxyz}	30.3^{pqrst}	31.3^{nopq}	32.7^{klmn}		
2% Coconut oil	27.3^{yz}	29.3^{stuvw}	29.7^{rstuv}	32.7^{klmn}		
1% Palm kernel oil	27.7^{xyz}	30.0^{qrstu}	31.0^{opqr}	33.0^{klmn}		
2% Palm kernel oil	27.3^{yz}	29.7^{rstuv}	30.3^{pqrst}	33.0^{jklm}		
1% Soy oil	27.7^{xyz}	30.0^{qrstu}	30.3^{pqrst}	32.0^{lmno}		

[1] A complete swine diet was first treated with 18 treatments and then inoculated with porcine epidemic diarrhea virus to mimic post-processing contamination. Once the inoculation was complete, the samples were analyzed on d 0, 1, 3, and 7 post inoculation for detectable porcine epidemic diarrhea virus RNA. Means presented are the interactive means of each treatment by analysis day and represented by n of 3. The PEDV negative treatment was analyzed on d 0 to verify that no PEDV was present in the feed. However, after this determination, it was not included in the statistical analysis as it was only analyzed on d 0.

[2] Cycle threshold required to detect the genetic material. A higher Ct value means less genetic material present.

[3] Sal Curb, Kemin Industries, Des Moines, IA.

[4] Medium chain fatty acid blend of 1:1:1 ratio of caproic, caprylic, and capric acids aerosolized into the mixer via an air atomizing nozzle.

[5] Medium chain fatty acid blend of 1:1:1 ratio of caproic, caprylic, and capric acids added directly into the mixer with no atomizing nozzle.

[6] 1:1 ratio of capric and lauric acids.

[7] Framelco, Raamsdonksveer, Netherlands.

[ab] Means within a column and row lacking a common superscript differ ($P < 0.05$).

Table 3.4 Main effects of day and treatment on Porcine Epidemic Diarrhea Virus PEDV detection using qRT-PCR

Item[1]	Ct[2]	SEM	*P* =
Analysis day[3]		0.130	<0.0001
d 0	29.5[a]		
d 1	31.9[b]		
d 3	33.1[c]		
d 7	34.6[d]		
Mitigation treatment[4]		0.262	<0.0001
PEDV negative	> 40.0		
PEDV positive	30.5[gh]		
0.325% Commercial formaldehyde [5]	33.5[d]		
1% MCFA (aerosolized)[6]	36.8[ab]		
1% MCFA (non-aerosolized)[7]	37.4[a]		
0.66 % Caproic acid	35.5[b]		
0.66% Caprylic acid	36.3[c]		
0.66% Capric acid	32.4[e]		
0.66 % Lauric acid	31.6[f]		
1% Capric:lauric acids[8]	32.3[e]		
0.3% FRA C12[9]	31.0[fg]		
1% Choice white grease	30.3[hi]		
1% Canola oil	30.1[hi]		
1% Coconut oil	30.6[gh]		
2% Coconut oil	29.8[i]		
1% Palm kernel oil	30.3[ghi]		
2% Palm kernel oil	30.1[hi]		
1% Soy oil	30.0[hi]		

[1] A complete swine diet was first treated with 18 treatments and then inoculated with porcine epidemic diarrhea virus to mimic post-processing contamination. Once the inoculation was complete, the samples were analyzed on d 0, 1, 3, and 7 post inoculation for detectable porcine epidemic diarrhea virus RNA. Means presented in the table are for the main effect of day and treatments.

[2] Cycle threshold required to detect the genetic material. A higher Ct value means less genetic material present. Cycle threshold of ≥ 40 was considered negative for presence of PEDV RNA.

[3] Main effect of analysis day on porcine epidemic diarrhea virus RNA detectability. Each analysis d is represented by an N of 51.

[4] Main effect of mitigation treatment on porcine epidemic diarrhea virus RNA detectability. Each treatment besides the PEDV negative treatment is represented by an N of 12. The PEDV negative treatment was analyzed on d 0 to verify that no PEDV was present in the feed. However, after this determination, it was not included in the statistical analysis as it was only analyzed on d 0. The PEDV negative mean is represented by and N of 3.

[5] Sal Curb, Kemin Industries, Des Moines, IA.

[6] Medium chain fatty acid blend of 1:1:1 ratio of caproic, caprylic, and capric acids aerosolized into the mixer via an air atomizing nozzle.

[7] Medium chain fatty acid blend of 1:1:1 ratio of caproic, caprylic, and capric acids added directly into the mixer with no atomizing nozzle.

[8] 1:1 ratio of capric and lauric acids

[9] Framelco, Raamsdonksveer, Netherlands.

[ab] Means within analysis day and mitigation treatment lacking a common superscript differ ($P < 0.05$).

Table 3.5 Effects of treatment on porcine epidemic diarrhea virus (PEDV) infectivity measured by pig fecal swabs and cecum content by qRT-PCR analysis

	PEDV N-gene Real Time-PCR, cycle threshold (Ct)						
		Fecal swabs					Cecum contents[4]
Item[1]	Feed Ct[2]	0 dpi[3]	2 dpi	4 dpi	6 dpi	7 dpi	7 dpi
d 0[5]							
PEDV negative	> 40.0	---[7]	---	---	---	---	> 45.0
PEDV positive	28.3	---	-- +	+++	+++	+++	22.2
d 1[6]							
PEDV positive	29.7	---	- ++	+++	+++	+++	20.9
0.325% Commercial formaldehyde [8]	33.0	---	---	---	---	---	> 45.0
1% MCFA (non-aerosolized)[9]	38.3	---	---	---	---	---	> 45.0
0.66 % Caproic acid	35.0	---	---	---	---	---	> 45.0
0.66% Caprylic acid	35.7	---	---	---	---	---	> 45.0
0.66% Capric acid	30.7	---	---	---	---	---	> 45.0
0.66 % Lauric acid	30.7	---	---	+++	+++	+-+	28.4
0.3% FRA C12[10]	30.7	---	---	+++	+++	+++	30.2
1% Canola oil	30.7	---	---	+++	+++	+++	20.3
1% Choice white grease	30.0	---	---	+++	+++	+++	15.3
1% Coconut oil	30.3	---	---	---	---	+-+	42.1
1% Palm kernel oil	30.0	---	---	+++	+++	+++	22.1
1% Soy oil	30.0	---	---	+++	+++	+++	24.0

[1]An initial tissue culture containing 10^6 $TCID_{50}$/mL PEDV was diluted to 10^5 $TCID_{50}$/mL PEDV. Each treatment was inoculated with the 10^5 $TCID_{50}$/mL PEDV resulting in 10^4 $TCID_{50}$/g PEDV inoculated feed matrix. Three feed samples per day and treatment were collected and diluted in PBS. The supernatant from each sample was then collected for pig bioassay. The supernatant was administered one time via oral gavage on d 0 to each of three pigs per treatment (10 mL per pig). Thus, the cecum contents are represented by a mean of 3 pigs per treatment. Pigs were inoculated at d 12 age.

[2] A cycle threshold (Ct of > 40) was considered negative for presence of PEDV RNA. Feed Ct analysis via qRT-PCR was carried out at Kansas State University. Values are from each analysis day by treatment interaction.

[3] Day post inoculation.

[4] A cycle threshold (Ct of > 45) was considered negative for presence of PEDV RNA. Cecum content analysis via qRT-PCR was carried out at Iowa State University Veterinary Diagnostic Laboratory at necropsy of the bioassay. Each value is represented by an n of 3 pigs.

[5] D 0 samples are represented from the d 0 analysis day and collected during the qRT-PCR analysis. The samples were collected and kept at -80°C until given to a 12 d old pig via oral gavage.

[6] D 1 samples are represented from the d 1 analysis day and collected during the qRT-PCR analysis. The samples were collected and kept at -80°C until given to a 12 d old pig via oral gavage.

[7] In each instance a (–) signals a negative pig in the bioassay and a (+) represents a positive fecal swab in the bioassay. Each day post inoculation within each treatment has three symbols within each row and column which represents one of the three pigs in each treatment.

[8] Kemin Industries, Des Moines, IA.

[9] Medium chain fatty acid blend of 1:1:1 ratio of caproic, caprylic, and capric acids added directly into the mixer with no atomizing nozzle.

[10] Framelco, Raamsdonksveer, Netherlands.

Chapter 4 - Determining the Minimum Inhibitory Concentration of Medium Chain Fatty Acids for generic Escherichia coli, Enterotoxigenic Escherichia coli, Salmonella Typhimurium, Campylobacter coli, and Clostridium perfringens

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ABSTRACT

OBJECTIVE

Determine the medium chain fatty acids (MCFA) minimum inhibitory concentration (MIC) for generic *Escherichia* coli, Enterotoxigenic *Escherichia* coli, *Salmonella* Typhimurium, *Campylobacter* coli, and *Clostridium* perfringens.

SAMPLE

Medium chain fatty acids of C6:0, C8:0, C10:0, and C12:0 and bacterial strains of generic *Escherichia* coli ATCC 25922, Enterotoxigenic *Escherichia* coli 3030-2, *Salmonella enterica* serotype Typhimurium ATCC 14028, *Campylobacte*r coli 7A 2016-1, and *Clostridium* perfringens 4026.

PROCEDURES

Minimum inhibitory concentrations were determined by modified microbroth dilution method using a 96 well microtiter plate with a concentration of 10^5 CFU/ml for each bacterial strain. Two products selected for quantification were mixed with a complete swine diet and inoculated with two concentrations (10^6 or 10^2 CFU/g of feed) of a Nal^R strain of Enterotoxigenic *Escherichia* coli (ETEC) for bacterial enumeration.

RESULTS

The MIC of MCFA varied among bacteria species. The lowest MIC of MCFA was 0.43% of a 1:1:1 blend of C6:0, C8:0, and C10:0 for *Campylobacte*r coli, 0.25% C12:0 for *Clostridium* perfringens, 0.60% 1:1:1 blend for generic *Escherichia* coli, 0.53% C6:0 for ETEC, and 0.40% C6:0 for *Salmonella* Typhimurium. Products with higher concentrations of C6:0 or C8:0 had a lower MIC in gram negative bacteria. When added to feed inoculated with ETEC, two MCFA-based products reduced ($P<0.05$) quantifiable bacteria in a linear manner.

CONCLUSION AND CLINICAL RELEVANCE

The inhibitory efficacy of MCFA varies among bacteria species. This suggest that MCFA mixture may provide a wider spectrum of bacterial control. As commercial products containing MCFA become available for livestock, it is important to consider the interaction between MCFA chain length and concentration on the potential to effectively mitigate various feed-based bacteria.

ABBREVIATIONS

MIC	Minimum inhibitory concentration
E. coli	generic *Escherichia* coli
ETEC	Enterotoxigenic *Escherichia* coli
S. Typhimurium	*Salmonella enterica* serotype Typhimurium
C. coli	*Campylobacte*r coli
C. perfringens	*Clostridium* perfringens
MCFA	Medium chain fatty acids
PBS	Phosphate buffered saline
CFU/ml	Colony forming unit per millimeter
CFU/g	Colony forming unit per g of feed
GC	Gas Chromatography

INTRODUCTION

Medium chain fatty acids (MCFA) have been demonstrated to significantly reduce problematic bacterial and viral contamination in animals, animal feed and feed ingredients.[1-7] Compared to

other feed additives, MCFA are unique in their potential mode of action. It is thought that the MCFA carry bacteriostatic and bactericidal properties by causing a destabilization of the bacterial double phospholipid bilayer membrane and causing the leakage of intracellular content.[2] It is also thought that the MCFA can acidify the cell by liberating H^+ ions, leading to cell death.[2] More recently, a 2% inclusion of a 1:1:1 ratio of C6:0, C8:0, and C10:0 reduced *Salmonella enterica* subsp. enterica serovar Typhimurium from 2.35 to 0.66 log CFU/g within 1 day.[3] The MCFA were also very effective on the initial inoculation day compared to the inoculated feed matrices containing no mitigation additives (2.35 vs. 5.45 Log CFU/g respectively).[3] However, there is a lack of information regarding which specific MCFA is the most effective, whether combinations of different MCFA exhibit additive effects, and what the optimal level of MCFA is that will impact various bacteria associated with animal production. This can be determined utilizing a minimum inhibitory concentration (MIC) benchtop assay, which identifies the lowest concentration of a treatment that prevents visible growth of a bacterium. Therefore, the objective of this study was to determine the minimum inhibitory concentration of specific MCFA and commercial products for *Campylobacter* coli, *Clostridium* perfringens, generic *Escherichia* coli, Enterotoxigenic *Escherichia* coli, and *Salmonella* Typhimurium as well as their potential application in feed as a reduction strategy.

MATERIALS AND METHODS

Bacterial inoculum

Bacterial strains of generic Escherichia coli (E. coli) ATCC 25922, Enterotoxigenic Escherichia coli (ETEC) 3030-2, and Salmonella enterica serotype Typhimurium (S. Typhimurium) ATCC 14028 were grown using Luria Bertani, Campylobacter coli (C. coli) 7A #2016-1 using Mueller-Hinton, and Clostridium perfringens (C. perfringens) 4026 using anaerobic Brain Heart Infusion

broth medium at 37°C for 24 h. For E. coli, ETEC, S. Typhimurium, and C. coli, 1 ml of bacterial inoculum was serially diluted using 9 ml of PBS to achieve one concentrations (10^5 CFU/ml) for each bacterial strain. For Clostridium perfringens, the bacterial concentration was adjusted to 0.5 McFarland Standards using fresh Brain Heart Infusion broth medium per Clinical and Laboratory Standards Institute recommendations.[8]

Experiment 1 MIC Determination of MCFA

For *E*. coli, ETEC, *S.* Typhimurium, and *C.* coli the compounds tested were C6:0[a], C8:0[a], C10:0[a], and a 1:1:1 blend of C6:0, C8:0, and C10:0. For *C.* perfringens, the compounds tested were C6:0, C8:0, C10:0, and C12:0[a].

The MIC were determined by the micro-broth dilution method as per Clinical Laboratory Standards Institute CLSI guidelines[8] in *E*. coli, *ETEC, S.* Typhimurium, and *C.* coli from 0.1% until an MIC was established, with a maximum tested level of 1.0%. The MIC was also determined using the same method for *C.* perfringens, with a maximum tested level of 2.0%. There were three replications per product and bacteria combination.

Experiment 2 MCFA Profiles and MIC Determination of Commercially-Based Products

The fatty acid profile of 21 commercially-based products was analyzed, with an emphasis on the MCFA concentration. The 24 products were, 1.) Product A[b], 2.) Product B[c] 3.) Product C[b], 4.) Product D[d], 5.) ProductE[d], 6.) ProductF[d], 7.) Product G[d], 8.) Product H[e] 9.) Product I[f], 10.) Product J[f], 11.) Product K[g], 12.) Product L[h], 13.) Product M[h], 14.) Product N[f], 15.) Product O[f], 16.) Product P[f], 17.) Product Q[f], 18.) Product R[f], 19.) Coconut Oil[g], 20.) Palm Oil[g], and 21.) Palm Kernel Oil[g]. Samples were analyzed according to procedures outlined by Sukhija and Palmquist[9]. From this analysis, Product A, B, G, H, and a commodity fat source (coconut oil)

were selected as having representative MCFA profiles for use in MIC assays. The profiles were selected based on products having the highest concentrations of C6:0 and C8:0 within the fatty acid profile and coconut oil because of its natural source of MCFA and medium chain triglycerides. The MIC were determined as described in Exp. 1 in *E*. coli, *ETEC*, *S.* Typhimurium, and *C.* coli from 0.1% until an MIC was established, with a maximum tested level of 5.0%. There were three replications per product and bacteria combination.

Experiment 3 Quantification of *Enterotoxigenic Escherichia* coli-inoculated feed after treatment with two commercially-based MCFA-containing products

Based on their lower MIC compared to other products tested in Exp. 2, Products A and B were selected as treatments to determine their reduction capacity in swine feed inoculated with ETEC. The strain of *ETEC* was first made resistant to 50 µl/ml nalidixic acid (Nal^R) antibiotic before being used for inoculation. A complete swine diet was either left un-inoculated and untreated, or mixed with 0.00, 0.25, 0.50, 1.00, or 2.00% Product A or B and inoculated with *ETEC*. For inoculation, 1 g of each feed sample was mixed with 1 ml of Nal^R *ETEC* at one of two concentrations (10^6 or 10^2 CFU per g of feed) of bacteria. The higher concentration was utilized for quantification of *ETEC* and the lower for detection. The 10 treatments were: 1.) control feed with no bacteria; 2). Control feed inoculated with bacteria and no addition of an additive; 3.) 0.25% Product A; 4.) 0.5%, Product A; 5.) 1.0%, Product A; 6). 2%. Product A; 7.) 0.5% Product B; 8.) 1.0% Product B; 9.) 2.0% Product B; and 10.) 4.0% Product B. The levels for each product were selected based on the results of Exp. 2. Product A was tested at a lower inclusion level in the feed because of the lower MIC value established in Exp. 2. Product B was then tested at higher inclusion levels because of the higher MIC value that was established in

Exp. 2. It was also determined that treatment 1 was confirmed to be negative of ETEC and was not included in the statistical model.

Samples were incubated at 37°C for 24 h. Then, 1 g of the incubated feed containing bacterial inoculum was suspended in 9 ml of PBS, serially diluted, and plated onto MacConkey agar containing nalidixic acid. The plates were incubated at 37°C for 24 h for bacterial enumeration using a standard plate count for viable cells. There were three replications per product and bacteria combination.

Statistical Analysis

Data from each MIC experiment were analyzed as a completely randomized design using PROC GLIMMIX in SAS[i] to evaluate the effect of each treatment within each bacterium. If the MIC value was greater than the detection limit of the analysis, the next logical inclusion level (increase in 0.1% inclusion) was utilized for the statistical analysis. For Exp. 3, the PROC GLIMMIX procedure of SAS[i] was utilized to evaluate linear and quadratic contrasts of increasing product levels. The coefficients for the unequally spaced linear and quadratic contrasts utilized in Exp. 3 were derived using the PROC IML procedure in SAS[i]. In all experiments, results for treatment criteria were considered significant at $P \leq 0.05$.

RESULTS

Experiment 1

The MIC of each MCFA in *C.* coli, *C.* perfringens, *E*. coli, *ETEC*, and *S.* Typhimurium, are presented in Table 1. The MIC for *C*. coli was lower ($P < 0.05$) in C6:0, C8:0, or the MCFA blend than in C10:0. In C. perfringens, the longer chain fatty acids were more effective with C12:0 and C10:0 providing the lowest ($P < 0.05$) MIC results with C12 being the most effective ($P < 0.05$) overall. Within generic E. coli, the 1:1:1 MCFA blend of C6:0, C8:0, and C10:0

provided the lowest ($P < 0.05$) MIC value followed by C6:0 and C8:0. Within Enterotoxigenic *E. coli*, C6:0 had a lower ($P < 0.05$) MIC than C8:0, which was still lower ($P < 0.05$) than either C10:0 or the MCFA blend, which were greater than the maximum tested value of 1%. In *Salmonella* Typhimurium, C6:0 resulted in an MIC similar ($P > 0.05$) to C8:0. However, C6 did differ ($P < 0.05$) from the blend. Again, no MIC was determined for C10:0 within *S.* Typhimurium.

Experiment 2

The fatty acid profile varied widely in the 21 commercially-based products (Table 2). Based on these analysis, Product A, B, F, G, and coconut oil were selected as candidate products for MIC determination in gram negative bacteria due to their high concentrations of C6:0 and C8:0. In *C.* coli, the MIC for Product B was lower ($P < 0.05$) than either Product F or G, with Product A being intermediate (Table 3). Product A and B had lower ($P < 0.05$) MIC in generic *E.* coli, ETEC, and *Salmonella* Typhimurium than other tested products. The MIC for coconut oil was not detected in any bacteria as it was greater than the maximum tested level of 5.0%.

Experiment 3

Due to their efficacy in the MIC determination, Product A and B were selected as treatments to determine their effect on detectable or quantifiable ETEC in feed. In the higher concentration of bacteria, Product A resulted in a linear decrease (linear, $P < 0.05$) in the number of quantifiable bacteria (Table 4). For Product B, as the inclusion level increased, the number of quantifiable bacteria quadratically decreased ($P < 0.05$). In the lower concentration of bacteria, Product A again resulted in a linear decrease (linear, $P < 0.05$) in the number of quantifiable bacteria (Table 5). However, in Product B no linear or quadratic response was observed ($P > 0.10$).

DISCUSSION

Our research supports previous findings where the MIC varies among MCFA and bacteria combinations.[10,11] For example, Skrivanova et al. showed an inclusion of C8:0 in two stains of *C.* perfringens (CCM 4435 and CNCTC 5459) resulted in MICs of 0.2% and 0.1% respectively.[12] However, when tested in *E.* coli CCM 3954 and 4225, the MIC of C8:0 and C10:0 were the same between the two bacterial strains within the same MCFA.[12] Meanwhile, the MIC in C6:0 was greater than the tested maximum inclusion of 0.5% for *E.* coli, *S.* typhimurium, *Salmonella* enteritidis, and *C.* perfringens.[12] This is in general agreement with our research. However, our findings extended the range of MIC detection, which allowed for the first time to the authors knowledge for the MIC values of different MCFA to be established for *E.* coli and *C.* perfringens.

Notably, the most effective MCFA was different when evaluating gram negative (*E*. coli, ETEC, *S*. Typhimurium, *C*. coli) compared to gram positive bacteria (*C*. perfringens). The membrane likely impacts the inhibitory process, as gram positive bacteria have thicker and more rigid peptidoglycan layers that provide increased protection for the phospholipid bilayer.[14] Furthermore, gram positive bacteria do not have the outer cell membrane like gram-negative bacteria do, so the mode of action of interfering with the cellular membrane may be different.

Based on the results from Exp. 1, we had hypothesized that feed additives rich in C6:0 would have greater efficacy than those rich in C10:0 or C12:0. This was confirmed, as the inclusion of C6:0 in *E. coli, ETEC*, and *S*. Typhimurium provided the lowest MIC values. Notably, there was also an apparent synergistic effect of including a blend of MCFA in *E. coli, S.* Typhimurium, and *C*. coli. In *C. coli*, the combination of the MCFA blend provided inhibitory benefit and could be the reason why Product B, F, and G presented an MIC value of

approximately 20% less than in the other bacteria tested. Products A, B, F, and G could contain other compounds within the product profile and were only selected based on the amount of MCFA within their profile. The other compounds that make up these products could also lead to the inhibitory effects that were observed. Also of note is that no MIC value was obtained for coconut oil. This could be because the MCFA in coconut oil are largely bound as triglycerides, which are not thought to interact with the cell membrane of gram negative bacteria without cleavage by lipase.

Results in Exp. 3 were based on the inclusion of each treatment into a complete swine diet and inoculated with two concentrations of ETEC. The higher concentration was utilized to capture the quantification of reduction associated with each treatment against the bacteria. The lower concentration was used as detection for the ETEC against each treatment. It should also be noted that Exp. 3 was a quantification not an MIC experiment. Results were similar to expectations as linear decreases of 0.62 and 0.54 log CFU/g of feed were noted for Product A in both the higher and lower bacteria concertation, respectively. However, this was not the case in Product B as no linear or quadratic decrease was noted at the lower concentration of ETEC tested.

This research extended previous knowledge to establish an MIC for four MCFA in five different bacteria. The effect of feeds or ingredients on MIC is still unknown, and necessary to consider as one applies this knowledge. Quantification was carried out in a complete feed matrix to determine the reduction each treatment provided against ETEC. However, further research is warranted to develop a complete feed MIC assay beyond quantifying bacteria to determine how a culture-based MIC method can be translated to a feed-based model.

In summary, the efficacy of MCFA varies among bacteria species. As commercial products containing MCFA become available for livestock feed manufacturers, it is important to consider the interaction between MCFA type and concentration on the potential for products to effectively mitigate various feed-based bacteria. It appears that C6:0 and C8:0 are more effective mitigants than C10:0 in gram negative bacteria, while C12:0 was the most effective in the gram-positive *C.* perfringens.

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FOOTNOTES

[a] Alfa Aesar, Ward hill, MA

[b] Nuscience Group, Ghent (Drongen), Belgium,

[c] Kemin Industries, Des Moines, IA.

[d] PMI Nutritional Additives, Arden Hills, Minnesota, USA

[e] Framelco, Raamsdonksveer, Netherlands

[f] Nutreco, Amersfoort, Netherlands

[g] ADM, Chicago, Illinois

[h] Cargill, Minneapolis, MN

[i] SAS, version 9.4, SAS Institute Inc, Cary, NC.

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TABLES

Table 4.1 Minimum inhibitory concentration of medium chain fatty acids in generic *Escherichia* coli, Enterotoxigenic *Escherichia* coli (ETEC), *Salmonella enterica* serotype Typhimurium, and *Clostridium* perfringens

Item[1]	MIC, %	SEM	*P*-Value
Campylobacter coli		0.047	0.0004
C6:0	0.50[b]		
C8:0	0.47[b]		
C10:0	0.90[a]		
1:1:1 Blend	0.43[b]		
Clostridium perfringens		0.030	< 0.0001
C6:0	1.65[a]		
C8:0	0.85[b]		
C10:0	0.70[c]		
C12:0	0.25[d]		
Generic *E*. coli		0.014	<.0001
C6:0	0.70[a]		
C8:0	0.85[b]		
C10:0[2]	> 1.00[c]		
1:1:1 Blend	0.60[d]		
Enterotoxigenic *E. coli*		0.024	<.0001
C6:0	0.53[c]		
C8:0	0.67[b]		
C10:0[2]	> 1.00[a]		
1:1:1 Blend[2]	> 1.00[a]		
Salmonella Typhimurium		0.050	<.0001
C6:0	0.40[c]		
C8:0	0.50[cb]		
C10:0[2]	> 1.00[a]		
1:1:1 Blend	0.60[b]		

[1] Minimum inhibitory concentration for C6:0, C8:0, C10:0, and a 1:1:1 blend of C6:0, C8:0, and C10:0 were tested in E. coli, ETEC, S. Typhimurium, and C. coli using a 96 well microtiter plate with a concentration of 10^5 CFU/ml for each bacterial strain. For C. perfringens, the compounds tested were C6:0, C8:0, C10:0, and C12:0 utilizing a 96 well microtiter plate with a concentration of 0.5 McFarland Standards for each well. Each value is represented by an N=3.

[2] Minimum inhibitory concentration was above the tested detection limit and therefore the next logical inclusion level (increase in 0.1% inclusion) was utilized for the statistical analysis.

[abcd] Means within a bacterial species lacking a common superscript differ ($P < 0.05$).

Table 4.2 Medium chain fatty acid profiles for the tested products (mg/g).

Item	Total analyzed fatty acids	C6:0	C8:0	C10:0	C12:0
Product A[1]	294.58	29.53	123.20	101.43	40.23
Product B[2]	1092.66	43.12	610.28	436.50	2.15
Product C[1]	123.07	12.35	51.42	42.28	16.85
Product D[3]	303.36	8.43	103.64	88.92	86.81
Product E[3]	369.33	9.02	123.38	105.61	111.06
Product F[3]	603.77	27.37	248.7	206.41	120.18
Product G[3]	494.34	0.98	227.13	188.00	74.50
Product H[4]	362.92	0.09	1.32	1.16	359.47
Product I[5]	349.54	2.19	159.32	131.10	56.71
Product J[5]	101.32	0.00	41.42	34.03	25.70
Product K[5]	402.37	0.20	128.21	99.30	122.71
Product L[7]	983.16	0.02	0.02	0.04	0.19
Product M[7]	520.80	3.78	40.87	31.21	227.83
Product N[5]	158.76	1.8	69.72	57.91	19.36
Product O[5]	145.57	1.74	68.08	56.43	18.56
Product P[5]	317.48	4.78	151.41	129.46	31.33
Product Q[5]	2.78	0.00	0.02	2.60	0.00
Product R[5]	314.01	0.69	101.44	83.01	90.15
Coconut Oil[6]	894.09	6.82	72.07	53.74	409.62
Palm Oil[6]	894.34	0.00	0.51	0.22	2.35
Palm Kernel Oil[6]	918.84	2.83	37.86	33.21	418.05

[1] Nuscience Group, Ghent (Drongen), Belgium
[2] Kemin Industries, Des Moines, IA.
[3] PMI Nutritional Additives, Arden Hills, Minnesota, USA
[4] Framelco, Raamsdonksveer, Netherlands
[5] Nutreco, Amersfoort, Netherlands
[6] ADM, Chicago, Illinois
[7] Cargill, Minneapolis, MN

Table 4.3 Minimum inhibitory concentration of commercially - based medium chain fatty acid based products in generic *Escherichia* coli, Enterotoxigenic *Escherichia* coli (ETEC), and *Salmonella enterica* serotype Typhimurium

Item[1]	MIC, %	SEM	*P*-Value
Campylobacter coli		0.629	0.0026
Product A[2]	1.20^{cd}		
Product B[3]	0.33^{d}		
Product F[2]	2.75^{bc}		
Product G[2]	3.33^{ab}		
Coconut oil[4,5]	> 5.0^{a}		
Generic *E. coli*		0.424	<.0001
Product A[2]	0.37^{c}		
Product B[3]	1.20^{c}		
Product F[2]	3.33^{b}		
Product G[2]	4.17^{ab}		
Coconut oil[4,5]	> 5.0^{a}		
Enterotoxigenic *E. coli*		0.309	< .0001
Product A[2]	0.33^{c}		
Product B[3]	1.30^{c}		
Product F[2]	3.83^{b}		
Product G[2]	4.33^{ab}		
Coconut oil[4,5]	> 5.0^{a}		
Salmonella Typhimurium		0.308	<.0001
Product A[2]	0.47^{c}		
Product B[3]	1.30^{c}		
Product F[2]	3.83^{b}		
Product G[2]	4.33^{ab}		
Coconut oil[4,5]	> 5.0^{a}		

[1] Minimum inhibitory concentration for products (Product A, B, F, G, and Coconut oil were tested in *E*. coli, *ETEC*, *S.* Typhimurium, and C. coli using a 96 well microtiter plate with a concentration of 10^5 CFU/ml for each bacterial strain. Each value is represented by an N=3.

[2] Nuscience Group, Ghent (Drongen), Belgium

[3] Kemin Industries, Des Moines, IA.

[4] ADM, Chicago, Illinois

[5] Minimum inhibitory concentration was above the tested detection limit and therefore the next logical inclusion level (increase in 0.1% inclusion) was utilized for the statistical analysis.

[abcd] Means within a bacteria species lacking a common superscript differ ($P < 0.05$).

Table 4.4 Effects of commercially-based products containing medium chain fatty acids on the growth of 10^6 CFU/g feed Enterotoxigenic Escherichia coli (ETEC)

Item[1]	Log CFU/g	SEM	Linear	Quadratic
Product A[2]		0.011	<.0001	0.9641
0.00%	5.44			
0.25%	5.37			
0.50%	5.24			
1.00%	5.15			
2.00%	4.81			
Product B[3]		0.017	<.0001	<.0001
0.00%	5.44			
0.50%	5.19			
1.00%	5.14			
2.00%	4.71			
4.00%	3.49			

[1] Product A and B were tested in a concentration of 10^6 CFU/g of feed ETEC in a complete swine diet in order to determine the growth of that bacteria using MacConkey agar containing nalidixic acid for bacterial enumeration

[2] Nuscience Group, Ghent (Drongen), Belgium

[3] Kemin Industries, Des Moines, IA.

Table 4.5 Effects of commercially-based products containing medium chain fatty acids on the growth of 10^2 CFU/g feed Enterotoxigenic Escherichia coli (ETEC)

Item[1]	Log CFU/g	SEM	Linear	Quadratic
Product A[2]		0.007	0.0060	0.1180
0.00%	2.95			
0.25%	2.93			
0.50%	2.93			
1.00%	2.95			
2.00%	2.91			
Product B[3]		0.012	0.1041	0.1579
0.00%	2.95			
0.50%	2.90			
1.00%	2.93			
2.00%	2.91			
4.00%	2.91			

[1] Product A and B were tested in a concentration of 10^2 CFU/g of feed ETEC in a complete swine diet in order to determine the growth of that bacteria using MacConkey agar containing nalidixic acid for bacterial enumeration

[2] Nuscience Group, Ghent (Drongen), Belgium

[3] Kemin Industries, Des Moines, IA.

Chapter 5 - Assessing the role of medium chain fatty acids as an alternative to Chlortetracycline

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ABSTRACT

An experiment was conducted to evaluate medium chain fatty acids (MCFA) as a potential alternative to chlortetracycline (CTC) in nursery pigs. One hundred entire male pigs (initially 6.4 ± 0.72 kg BW and weaned at 22 days of age) were used in a 29-day disease challenge study. Pigs were allowed 5 acclimation days, followed by 2 days of disease challenge with enterotoxigenic β-hemolytic *Escherichia. coli*, serotype O149:K91: K88 (ETEC). After the challenge, pigs were allotted to a diet with 1 of 5 treatments: 1) Control with no additives, 2) 400 g/ton CTC (Chlortet 200G, Eco Animal Health, London, United Kingdom), 3) 1.08% of a 1:1:1 blend of C6:0, C8:0, and C10:0 (Nuscience Group, Drongen, Belgium), 4) 3.93% developmental Product A (Nuscience Group, Drongen, Belgium), and 5) 1.04% developmental Product B (Kemin Industries, Des Moines, IA). Treatments 3, 4, and 5 were included at rates to derive a 1% MCFA concentration in finished feed. Pigs were fed treatment diets for 14 days following the disease challenge to mimic a therapeutic dose of CTC and fed a common diet from d 14 to 21. There was no difference ($P > 0.10$) of dietary treatment on growth performance form d 0 to 7 or d 14 to 21. From d 7 to 14, pigs fed diets supplemented with CTC, 1:1:1 blend, or Product B had improved ($P < 0.05$) G:F compared to those fed the control diet, with pigs fed diets with Product A being intermediate. A treatment × day interaction for the ETEC fecal shedding was observed ($P < 0.05$), which was driven by pigs fed diets with CTC having decreased ($P < 0.05$) fecal shedding on d 7 than 14, while those fed diets with Product B having greater ($P < 0.05$) fecal ETEC shedding on d 1 than d 14. While other disease markers, such as fecal score, plasma urea nitrogen, and haptoglobin, decreased ($P < 0.05$) with time, they were not affected ($P > 0.05$) by dietary treatment. In conclusion, supplementing ETEC-challenged nursery pigs with MCFA-based dietary treatments led to similar growth performance as a therapeutic dose of 400 g/ton of

CTC. Further research is needed to confirm the mode of action, most effective MCFA or combination, and effective dose of medium chain fatty acids in ETEC-challenged pigs.

Key words: chlortetracycline, Enterotoxigenic *Escherichia coli*, medium chain fatty acids, pig

INTRODUCTION

There is increasing consumer and regulatory pressure to reduce feed-based antibiotic use in food animals (Center for Disease Control, 2013; Landers et al., 2012). As stewards of animal health, pork producers are challenged to reduce their reliance on antimicrobials, particularly when pigs are faced with a disease challenge. Antibiotics, such as Chlortetracycline (CTC), are highly effective at reducing mortality and morbidity of nursery pigs challenged by disease, and their removal from the diet leaves pork producers concerned about both profitability and animal well-being (Elliott et al., 1964; Cromwell, 2002; Thacker, 2013; Fouhse et al., 2016). Currently, the industry is implementing the Veterinary Feed Directive regulation, which removes the growth promotion label and requires veterinary oversight for judicious use of over-the-counter drugs (Food and Drug Administration, 2015). There is concern regarding the use of CTC which represents 61% of the volume of highly-important antibiotics and 42% of the total antibiotic use in swine feed (Apley et al., 2012). Even with these regulations in place, the future potential use of antibiotics in feed is unknown. Thus, pork producers are looking for alternatives to medically-important antibiotics, particularly those used therapeutically at weaning to maintain animal health. Several classes of feed additives have antimicrobial properties, including probiotics, prebiotics, enzymes, acidifiers, plant extracts, and nutraceuticals (Thacker, 2013).

One such alternative includes medium chain fatty acids (MCFA), specifically C6:0, C8:0, and C10:0. These MCFA have recently demonstrated mitigation potential against PEDV and bacteria (Cochrane et al., 2016; Dee et al., 2016; Cochrane et al., 2017;). A 2%, and 1%

inclusion of a 1:1:1 ratio of C6:0, C8:0, and C10:0 as well as the inclusion of 0.66% of the C6:0, C8:0, and C10:0 individually, led to a reduction of detectable PEDV RNA and prevent infection within a swine bioassay (Cochrane et al., 2018). This demonstrates the potential for MCFA to work both *in vivo* and *in vitro*. The same effect has also been noted in bacterial species as a 2% inclusion of the 1:1:1 blend utilized by Cochrane et al., (2016) led to a 2-log reduction of *Salmonella* Typhimurium inoculated feed ingredients. More recently, minimum inhibitory concentrations (MIC) of MCFA against Campylobacter coli, Clostridium perfringens, generic Escherichia coli, Enterotoxigenic Escherichia coli, and *Salmonella* Typhimurium were established (Swanson et al., 2018). However, it was determined that MIC of MCFA varied among bacterial species (Swanson et al., 2018). The ability of MCFA to mitigate bacterial species, including enterotoxigenic *E. coli,* and server as a antimicrobial replacement in swine diets is unknown. Therefore, the objective of this study was to compare the efficacy of MCFA vs. a therapeutic dose of chlortetracycline supplementation in feed for Enterotoxigenic *Escherichia coli*-challenged pigs.

MATERIALS AND METHODS

This study was approved by the Animal Ethics Committee of Murdoch University, Murdoch Western Australia (R2969/17).

Animals and Housing

A total of 100 entire male pigs (Large White × Landrace: initially 6.4 ± 0.72 kg weaned at an average of 22 days of age) were used in a 29-d disease challenge study to evaluate MCFA as a potential alternative to CTC. Pigs were obtained from a commercial operation on the day of weaning and transported to the Murdoch University research facility. Upon arrival, pigs were

weighed, allotted to pens based on body weight, and fecal rectal swabs were collected for baseline levels of β-hemolytic *Escherichia. Coli* (ETEC). Pens were equipped with a 5-hole, dry self-feeder, and a pan waterer to provide *ad libitum* access to feed and water. Pigs were allowed 5 days of acclimation on a corn and soybean meal common diet (d -7 to -2; Table 5.1). On d -2, pigs were weighed, and randomly allotted to dietary treatments based on BW with 5 pigs per pen and 4 pens per treatments. Blood was collected from 2 random pigs per pen to establish baseline blood metabolites for plasma urea nitrogen, C-reactive protein, and haptoglobin.

Infection Procedures

All pigs were oral inoculated with ETEC acccording to Heo et al., (2009) on d -2 and d -1. Briefly, a strain of enterotoxigenic β-hemolytic *E. coli*, serotype O149:K91:K88 (variants STa and STb), was grown, selected, and incubated. The resultant pellet was suspended, placed into gelatin capsules, and held on dry ice until use. On day -2 and -1, each pig received two capsules of inoculum, for a total of 1600 μL. Enterotoxigenic β-hemolytic *E. coli* concentration of the capsules on d -2 was 2.56×10^{9} CFU/mL, and on d -1 was 8.80×10^{8} CFU/mL.

Experimental Design and Treatments

On d 0, the common diet used during acclimation was changed to include one of the following treatments: 1) no additives (control); 2) 440 mg/kg CTC (Chlortet 200G, Eco Animal Health, London, United Kingdom),; 3) 1.08% of a 1:1:1 blend of C6:0, C8:0, and C10:0 (contained 32.7% C6:0, 33.7% C8:0, 33.3% C10:0 Nuscience Group, Drongen, Belgium); 4) 3.93% developmental Product A (contained 2.95% C6:0, 12.3% C8:0, 10.1% C10:0; Nuscience Group, Drongen, Belgium), and 5) 1.04% developmental Product B (contained 3.9% C6:0, 54.2% C8:0, 38.5% C10:0; Kemin Industries, Des Moines, IA). Treatments 3, 4, and 5 were included at rates to derive a 1% MCFA concentration in finished feed. Dietary treatments were

fed for 14 d, then pigs were fed a common commercial pelleted diet (Farmyard Pig Weaner, Weston Milling, Perth Western Australia) from d 14 to 21. The commercial pelleted diet contained 20% CP, 1.2% Lysine, 14.5 MJ/kg digestible energy, 0.85% calcium, and no added zinc oxide or antibiotics. Diet samples were collected and analyzed for DM, CP, crude fiber, EE, Ca, and P by Agrifood technology (Bibra Lake Western Australia) (Table 5.2). Chlortetracycline levels in the feed were analyzed at Symbio Laboratories (Sydney, Australia). Diets were also analyzed for MCFA concentration by Fatty Acid Methyl Estes Gas Chromatography at the Department of Primary Industries (Wagga Wagga, New South Wales Australia).

Clinical Disease Characterization

Pigs and feeders were weighed on d -2, 0, 7, and 14 of the trial to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F). Pigs were evaluated daily for fecal scores using the following systems: 1) firm, well-formed feces, 2) soft formed feces, 3) soft and loose shape, or 4) watery liquid consistency. In accordance with animal ethics application (R2969/17), if a pig exhibited a diarrhea score of 4 for 48 hours, it was treated with Moxylan (Amoxycillin, Jurox, Rutherford, New South Wales Australia). Each pig treated received three doses of the Moxylan. One pig in each of the control, 1:1:1 MCFA blend, and Product A groups was treated, while 2 pigs were treated in the CTC and Product B groups. Fecal shedding of ETEC was evaluated according to Heo et al., (2009) by fecal swabs collected on d -7, -2, 0, 1, 3, 7, and 14. Swabs were plated using a 5-zone streaking method, incubated overnight, and scored from 0 to 5 with 0 representing no growth and 5 representing growth out to the fifth section. Blood samples were collected on d -2, 7, and 14 from 2 pigs per pen according to Stensland et al., (2015). Briefly, samples were collected via jugular vein puncture into a lithium heparin tube. Tubes were centrifuged at 3000 × g for 10 min at room temperature, plasma

collected, and stored at -20°C until analyzed for plasma urea nitrogen (PUN), haptoglobin, and C-reactive protein (C-RP). The PUN was determined using a Beckman Coulter/Olympus Reagent Kit (OSR6134) and haptoglobin by In-House Method NTM-62 (Eckersall et la., 1991). Both PUN and Haptoglobin analysis were performed on an Olympus Clinical Chemistry Analyzer. The PUN and Haptoglobin were analyzed by Animal Health Labs (Department of Primary Industries and Regional Development, South Perth, Western Australia). C-reactive protein was analyzed using a DuoSet ELISA (R&D systems for Porcine C-Reactive Protein/CRP cat No: DY2648) and analyzed at Murdoch University (Murdoch Western Australia).

Statistical Analysis

Data was analyzed as a completely randomized design with pens randomly alloted to treatment based on BW. Pen was considered the experimental unit. Fecal scores and enterotoxigenic β-hemolytic *E.* coli fecal shedding scores were analyzed as repeated measures across day. Unequal spaced analysis days for enterotoxigenic β-hemolytic *E.* coli fecal shedding scores were accounted for within the statistical model. All possible pairwise comparisons were protected by the Tukey-Kramer adjustment. Results for treatment criteria were considered significant at $P \leq 0.05$ and marginally significant from $P > 0.05$ to $P \leq 0.10$. Data were analyzed using the GLIMMIX procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NC).

RESULTS

During the infection portion of the study, from d -2 to 0, pigs had an ADG of 0.16 kg, ADFI of 0.20 kg, and G:F of 0.78. Dietary treatment did not impact ($P > 0.10$) body weight, ADG, ADFI, and G:F from d 0 to 7 (Table 5.3). From d 7 to 14, pigs fed diets supplemented with CTC, 1:1:1 blend, or Product B all had greater ($P < 0.05$) G:F than pigs fed the control diet, with pigs fed Product A being intermediate ($P > 0.10$). This led to pigs being fed CTC or Product

B having improved ($P < 0.05$) G:F during the entire treatment phase, from d 0 to 14. This effect continued after treatment diets ended, where pigs fed diets containing Product B had marginally significant greater ($P < 0.10$) G:F than those fed the control diet from d 0 to 21.

A treatment × day interaction for the ETEC fecal shedding was observed ($P < 0.05$; Table 5.4). This was driven by pigs fed diets with CTC having decreased ($P < 0.05$) fecal shedding on d 7 than 14, while those fed diets with Product B having greater ($P < 0.05$) fecal ETEC shedding on d 1 than d 14. While other disease markers, fecal score, PUN, and haptoglobin, decreased ($P < 0.05$) with time, there was no evidence for ($P > 0.10$) effects of dietary treatment or the interaction between treatment and time. A decrease ($P < 0.05$) in fecal scores (2.6, 1.9, and 1.4) was notated as time increased on d 0, 3, and 7 respectively with no further reduction beyond d 6. A decrease ($P < 0.05$) in PUN (2.8 to 2.2) and haptoglobin (0.7 to 0.1) were noted from on d -2 to 14 respectively. No evidence for C-RP was observed in the experiment ($P > 0.10$).

DISCUSSION

Medium chain fatty acids have previously been shown to improve growth performance in weanling pigs (Hanczakowska et al., 2011; Zentek et al., 2013; Mohana Devi and Kim, 2014; Hanczakowska et al., 2017). This experiment did not show discernable differences in ADG, but demonstrated that diets containing high amounts of C6:0, C8:0, and C10:0 in the specific combination and concentrations utilized in the products may be as useful as CTC in maintaining feed efficiency during an immune challenge. While they have a similar effect, the modes of action between CTC and MCFA likely differ. Chlortetracyclines transvers the outer membrane of gram negative bacteria through the OmpF and OmpC porin channels as positively charged cations (Chopra and Roberts, 2001). The antibiotic complex is then attracted by Donnan potential across the outer membrane leading to accumulation in the periplasm where it

dissociates, binds with ribosomes and inhibits bacterial protein synthesis (Chopra and Roberts, 2001). Medium chain fatty acids are thought to act as nonionic surfactants which incorporate themselves into the bacterial cell membrane or diffuse through the cell membrane (Desbois and Smith, 2010; Kim and Rhee, 2013). This in turn creates pores and changes the membrane permeability to allow the leakage of intercellular content that ultimately leads to bacterial cell death (Desbois and Smith, 2010; Kim and Rhee, 2013).

The current study supports previous research that reported less than 1% of C6:0 or C8:0 can inhibit ETEC growth *in vitro* and also showed that 0.33% Product A or 1.30% Product B completely limited ETEC growth *in vitro* (Swanson et al., 2018). A notable difference between Swanson et al. (2018) is certainly that an *in vivo* model was used in the current experiment, but also that the efficacy is tested in feed versus broth media. In the current experiment, the sum of C6:0 + C8:0 + C10:0 concentration was held constant in the MCFA treatments, yet Product B showed the greatest efficacy. This could potentially be explained by the unique MCFA profile that was utilized for Product B compared to Product A and the MCFA blend. As noted in the analyzed feed samples, the treatments had varying levels of each MCFA within the complete feed and could help to potentially explain the observed responses.

Both Products A and B are products currently under commercial development, as opposed to the purified 1:1:1 MCFA blend. As a result, Products A and B could contain organic acids and other compounds that explain slightly differing results among MCFA sources. These additional compounds may change the pKa of products and impact the ability of MCFA to approach the ETEC membrane. However, additional research is needed to confirm the role of additional additives on the efficacy of MCFA.

This research is important as it establishes a nursery pig feed additive that offers equivalent growth performance potential as CTC during ETEC challenge. Other researchers have reported similar G:F responses when supplementing diets with a similar blend of MCFA outside a health challenge (Thompson et al. 2018). While genetics and environmental conditions between the two experiments differ, the data suggest that MCFA may have efficacy both during and outside an immune challenge.

The G:F improvement from pigs fed Product B may be partially explained by the fact that the same pigs had reduced fecal shedding within the treatment on d 14. Interestingly, the effect was reversed for pigs fed CTC, where fecal shedding actually increased form d 7 to 14. This may be due to ETEC resistance to CTC, which was confirmed after the trial was complete. The minimal effect of MCFA supplementation on fecal score was similar to findings from Mohana Devi and Kim, (2014), who found no treatment effect in swine diets containing 0.2% MCFA. Though Mohana Devi and Kim (2014) did not include a disease challenge, so it is logical that fecal score was not impacted over time, where the current study had improved fecal scores as pigs recovered from the ETEC challenge.

As with prevoius research (Heo et al., 2009; Stenslend et al., 2015), plasma urea nitrogen and haptoglobin decreased over time in pigs experimentally infected with the same strain of enterotoxigenic β-hemolytic *E.* coli (Heo et al., 2009; Stenslend et al., 2015). This decrease could potentially be caused by a decrease in the microbial fermentation of nitrogenous compounds in the large intestine (Kim et al., 2008). Plasma urea nitrogen is a marker for increased microbial production of NH_3-N (Younes et al., 1998), and is a biomarker of intestinal health in the post-weaning period for pigs (Awati et al., 2007). Meanwhile, haptoglobin is a positive acute phase

protein that would be expected to increase when an infection or inflammation is present (Stensland et al., 2015).

Finally, there was no evidence of a treatment effect on C-Reactive protein within this study. One of the reasons that this could have occurred is because C-RP rises and falls more rapidly and dramatically than many other acute-phase proteins (Du Clos and Mold, 2004). This makes it a useful marker to follow clinical disease and response to a treatment (Du Clos and Mold, 2004). However, the blood sampling on d -2, 7, and 14 may have been too far apart to measure the acute phase response of C-RP. Future research should evaluate the effectiveness of MCFA and ETEC infection closer to the time of inoculation.

In conclusion, MCFA-based treatments provided similar response as CTC to pigs challenged with enterotoxigenic β-hemolytic *Escherichia coli*. Therefore, further research regarding MCFA supplementation in feed is warranted to confirm the mode of action and ideal inclusion levels in pigs challenged with ETEC and other disease challenge models.

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TABLES AND FIGURES

Table 5.1 Formulated composition of the basal diet (as-fed basis)

Item[1]	Control
Ingredient, %	
Corn	55.00
Soybean meal (48%)	21.87
Fish Meal	4.00
HP 300	5.0
Whey	10.00
Soybean oil	1.00
Monocalcium phosphate	1.20
Limestone	1.23
Sodium chloride	0.60
L-Lys-HCl	0.35
DL-Met	0.18
L-Thr	0.18
L-Trp	0.03
L-Val	0.05
Trace mineral and vitamin premix[2]	0.10
Total	100.00
Calculated analysis	
Standard ileal digestible (SID) amino acids, %	
Lys	1.35
Ile:lys	59
Leu:lys	119
Met:lys	37
Met & Cys:lys	58
Thr:lys	65
Trp:lys	19
Val:lys	68
SID lysine:ME, g/Mcal	4.43
ME, kcal/lb	1,532
Total lysine, %	1.50
CP, %	22.1
Ca, %	0.79
P, %	0.75
Available P, %	0.47

[1] The basal diet was fed to all pigs from d -7 to 0 and for the control group of pigs. The basal diet was also used for each treatment diet in which corn was replaced with the respective treatments. The CTC treatment was included at 400g/ton, 1:1:1 MCFA blend at 1.1%, Product A at 3.9%, and Product B at 1.0%. In each instance, the same percentage of corn was removed and replaced with the addition of the treatments. The 1:1:1 MCFA blend, Product A, and Product b were included to reach a total MCFA inclusion level of 1.0%

[2] BJ Grower (Biojohn Pty Ltd, Perth, WA, Australia) Provided the following nutrients (per kg of premix) Vitamins: A 5300 IU, D3 1000 IU, E 46.67g, K 1.33 g, B1 1.33 g, B2 3.33 g, Niacin 16.67 g, B5 28.72 g, B6 1.67 g, folic acid 0.67 g, B12 13.33 mg, and biotin 66.67 mg. Minerals: Co 0.33 g (as cobalt sulfate), Cu 13.33 g (as copper sulfate), iodine 0.67 g (as potassium iodine), iron 40 g (as ferrous sulfate), Mn 26.67 g (as manganous oxide), Se 0.2 g (as sodium selenite), Se inorganic 0.07g (as selenosource), Se organic 0.13 g (as selenosource), and Zn 66.67 g (as zinc sulphate).

Table 5.2 Analyzed diet composition (as-fed basis)

Analyzed composition, %[1]	Control	CTC[2]	1:1:1 MCFA Blend[3]	Product A[4]	Product B[5]
DM	91.3	91.3	91.0	90.0	90.7
CP	21.6	21.8	20	22.1	22.2
Crude Fiber	1.9	2.1	1.7	1.6	1.9
Total Fat	3.4	3.4	3.4	3.2	3.5
Ca	0.93	1.50	0.87	0.86	1.10
P	0.76	0.72	0.74	0.79	0.74
CTC	0.00	0.04	0.00	0.00	0.00
C6:0	0.01	0.01	0.28	0.09	0.01
C8:0	0.02	0.01	0.33	0.38	0.44
C10:0	0.03	0.03	0.30	0.32	0.35
Total MCFA[6]	0.06	0.05	0.91	0.79	0.80

[1] Complete diet samples were collected following feed manufacture, subsampled, and submitted to Agrifood Technology (Bibra Lake Western Australia for proximate analysis. The samples were also analyzed for MCFA concentration at the Department of Primary Industries (Wagga Wagga New South Wales Australia)

[2] Formulated to contain the regulatory limit of chlortetracycline (400g/ton). Analyzed value for the CTC diet was 356 g/ton or 0.0356%.

[3] 1:1:1 ratio of C6:0, C8:0, and C10:0 formulated to contain 1% of MCFA in the complete diet. Each fatty acid supplied from Nuscience Group, Ghent (Drongen), Belgium.

[4] Formulated to contain 1% of MCFA in the complete diet (Nuscience Group, Ghent (Drongen), Belgium).

[5] Formulated to contain 1% of MCFA in the complete diet (Kemin Industries, Des Moines, IA).

[6] Sum of analyzed C6, C8, and C10 medium chain fatty acids.

Table 5.3 Effects of Chlortetracycline and MCFA Treatments on Nursery Pig Performance.

Item; [1]	Control	CTC	1:1:1 MCFA Blend[2]	Product A[3]	Product B[4]	SEM	*P* =
BW, kg[5]							
d 0	7.5	7.3	7.4	7.4	7.3	0.203	0.9669
d 7	10.5	10.5	10.3	10.6	9.8	0.383	0.6756
d 14	14.5	15.0	14.7	14.3	14.0	0.474	0.6251
d 21	18.7	19.0	19.2	18.8	18.3	0.522	0.7690
d 0 to 7							
ADG, kg[6]	0.44	0.45	0.41	0.44	0.36	0.029	0.2456
ADFI, kg[7]	0.44	0.46	0.43	0.45	0.37	0.034	0.3380
G:F[8]	1.00	0.98	0.94	1.00	1.00	0.033	0.6910
d 7 to 14							
ADG, kg	0.56	0.64	0.63	0.53	0.59	0.030	0.0848
ADFI, kg	0.88	0.76	0.83	0.74	0.71	0.044	0.0740
G:F	0.64^{a}	0.85^{b}	0.77^{b}	0.72^{ab}	0.84^{b}	0.031	0.0009
d 14 to 21							
ADG, kg	0.59	0.57	0.64	0.65	0.61	0.034	0.5341
ADFI, kg	0.86	0.89	0.90	0.91	0.88	0.033	0.8753
G:F	0.69	0.64	0.70	0.72	0.70	0.023	0.2278
d 0 to 14							
ADG, kg	0.50	0.55	0.52	0.49	0.48	0.024	0.3153
ADFI, kg	0.66	0.61	0.63	0.60	0.54	0.035	0.1746
G:F	0.76^{a}	0.90^{b}	0.83^{ab}	0.82^{ab}	0.90^{b}	0.026	0.0076
d 0 to 21							
ADG, kg	0.53	0.56	0.56	0.55	0.52	0.020	0.5065
ADFI, kg	0.73	0.70	0.72	0.70	0.65	0.029	0.3408
G:F	0.73^{x}	0.79^{xy}	0.78^{xy}	0.78^{xy}	0.80^{y}	0.028	0.0784

[1] A total 100 entire male pigs (Large White × Landrace: initially 6.4 ± 0.72 kg weaned at an average of 22 days of age) were used in a 29 - day disease challenge study to evaluate MCFA as a potential antibiotic alternative to CTC. The pigs were acclimated for 6 days (d -7 to -2) before receiving 2 capsules of ETEC inoculum each on d -2 and -1 for a total of 4 capsules. During the acclimation phase and inoculation phase, pigs received a basal diet. Treatment diets were then fed from d 0 to 14 and then placed onto a commercial pelleted diet for the final 7 grow out days.

[2] 1:1:1 ratio of C6:0, C8:0, and C10:0. Each fatty acid supplied from Nuscience Group, Ghent (Drongen), Belgium.

[3] Nuscience Group, Ghent (Drongen), Belgium.

[4] Kemin Industries, Des Moines, IA.

[5] Body weight.

[6] Average Daily Gain.

[7] Average Daily feed intake.

[8] Gain to Feed ratio.

[ab] Means within a row lacking a common superscript differ ($P < 0.05$). All possible pairwise comparisons were protected by the Tukey-Kramer adjustment.

[xy] Means within a row lacking a common superscript differ ($P \leq 0.10$). All possible pairwise comparisons were protected by the Tukey-Kramer adjustment.

Table 5.4 Interactive means of Treatments × Day on Enterotoxigenic E. coli fecal shedding.

Item;[1]	Control	CTC	1:1:1 MCFA Blend[2]	Product A[3]	Product B[4]	SEM	*P* =
Pre-inoculation[5]						0.3020	<.0001
d -7	0.00	0.25	0.15	0.20	0.00		
Inoculation[6]							
d -2	0.10	0.10	0.00	0.05	0.20		
Treatment phase							
d 0	0.65^{abcde}	1.00^{abcde}	1.10^{abcde}	1.25^{abcde}	1.05^{abcde}		
d 1	0.75^{abcde}	1.30^{abcde}	1.05^{abcde}	1.55^{abcde}	1.80^{ac}		
d 3	0.90^{abcde}	0.45^{abcde}	1.25^{abcde}	0.35^{abcde}	0.95^{abcde}		
d 7	0.80^{abcde}	0.10^{bcd}	0.10^{abcde}	0.25^{abcde}	1.25^{abcde}		
d 14	0.90^{abcde}	1.40^{ae}	0.70^{abcde}	0.70^{abcde}	0.05^{bde}		

[1] Fecal rectal swabs were collected on each pig on d -7, -2, 0, 1, 3, 7, and 14 by inserting a cotton swab into the anus of the pig. The swabs were then plated using a 5-zone streaking method in which each swab was streaked onto zone 1 of the plate. A wire loop was then utilized to streak from zone 1 to zone 5. The wire loop was sanitized before moving to the next zone. Plates were incubated overnight at 37°C. The plates were scored on a scale of 0 to 5 according to the number of sections containing viable hemolytic E. coli where 0 was no growth and 5 was growth out to the fifth section. Statistical analysis was only completed on samples taken during the treatment phase. Means presented are the interaction of treatment and day.
[2]1:1:1 ratio of C6:0, C8:0, and C10:0. Each fatty acid supplied from Nuscience Group, Ghent (Drongen), Belgium.
[3] Nuscience Group, Ghent (Drongen), Belgium.
[4] Kemin Industries, Des Moines, IA.
[5] Baseline levels were taken for each pig on the day of arrival.
[6] Baseline levels were taken for each pig prior to receiving the ETEC inoculum.
[abcde]Means within a row and column lacking a common superscript differ ($P < 0.05$). All possible pairwise comparisons were protected by the Tukey-Kramer adjustment.