CHEMICAL MITIGATION OF MICROBIAL PATHOGENS IN ANIMAL FEED AND INGREDIENTS

by

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B.A., Franklin College, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTERS OF SCIENCE

Department of Grain Science & Industry
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Manhattan, Kansas

2015

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Abstract

Feed mill biosecurity is a growing concern for the feed industries, especially since the entry of Porcine Epidemic Diarrhea Virus (PEDV) to the United States. Porcine Epidemic Diarrhea Virus (PEDV) is primarily transmitted by fecal-oral contamination. However, research has confirmed swine feed and ingredients as potential vectors of transmission, so strategies are needed to mitigate PEDV in feed. The objective of the first experiment was to evaluate the effectiveness of various chemical additives to prevent or mitigate PEDV in swine feed and ingredients that had been contaminated post-processing. Time, formaldehyde, medium chain fatty acids, essential oils, and organic acids all enhance the degradation of PEDV RNA in swine feed and ingredients, but their effectiveness varies within matrix. Notably, the medium chain fatty acids were equally as successful at mitigating PEDV as a commercially-available formaldehyde product.

Salmonella is also another potential feed safety hazard in animal feed ingredients. Thermal mitigation of *Salmonella* in ingredients and feed manufacturing is effective, but it does not eliminate the potential for cross contamination. Therefore, the objective of the second experiment was to evaluate the effectiveness of chemicals to mitigate *Salmonella* cross-contamination in rendered proteins over time. Both chemical treatment and time reduced *Salmonella* concentrations, but their effectiveness was again matrix dependent. Chemical treatment with medium chain fatty acids or a commercial formaldehyde product was most effective at mitigating *Salmonella* in rendered protein meals.

The final experiment was conducted to evaluate the effectiveness of a dry granular acid, sodium bisulfate (SBS; Jones-Hamilton, Co., Waldridge, OH), to mitigate contamination of *Salmonella* in poultry feed. A surrogate organism, *Enterococcus faecium,* was utilized for this research in order to evaluate the effectiveness of SBS. Thermal processing, SBS concentration, and time all

impacted biological pathogen levels in poultry diets, and including a dry granular acid may be an effective method to reduce pathogen risk. However, the most significant reduction of Enterococcus faecium was due to thermal mitigation. Notably, pelleting reduced *Enterococcus faecium* by 2-3 logs on day 0. In summary, both thermal processing and chemical inclusion can be used to reduce the risk of microbial pathogens in feed.

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Acknowledgements

I would like to thank Jones-Hamilton Co., Anitox Corp., Fats and Proteins Research Foundation, National Pork Board, and Iowa State University's Veterinary Diagnostic Laboratories for their contributions to this research. I would also like to recognize Drs. Cassandra Jones, Jason Woodworth, Steve Dritz, Charles Stark, and Anne Rigdon-Huss for their support and guidance throughout this research. Finally, I would like to thank Anita McDiffett for her help in assisting with the everyday aspects of graduate school.

Dedication

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

Chapter 1 - Systematic Approach to Microbiological Feed Mill Biosecurity in Swine

Roger Cochrane, Steve Dritz, Jason Woodworth, Charles Stark, Anne Huss, and Cassandra Jones

Summary

Feed mill biosecurity is a growing concern for the swine and feed industries, especially since the entry of Porcine Epidemic Diarrhea Virus (PEDV) to the US and the fact that it has been proven that feed is a possible vector for transmission. The development of a biosecurity plan can help protect swine herd health and limit economic loss from diseases, such as PEDV, and other microbial pathogens like *Salmonella*. An effective biosecurity plan should be detailed and contain hazard controls at each part of the manufacturing process. . According to the FDA, "a hazard is a biological chemical or physical agent that is reasonably likely to cause illness or injury in the absence of its control."[1] Hazards can be introduced by ingredients, manufacturing equipment, or people among others, so controls at each point of manufacturing must address the prevention or mitigation of hazards from each source. Thus, the biosecurity plan should contain ingredient specifications, sampling methods, analytical procedures, receiving guidelines, equipment cleanout SOPs, flushing or sequencing orders, production parameters, load-out procedures, and sanitation expectations. A biosecurity assessment may be a useful tool to evaluate the successful adaptation of a biosecurity plans and demonstrate areas where new emphasis is needed. If biosecurity measures are successfully implemented, product safety should be maximized and the risk of microbiological pathogens in animal feed reduced.

Introduction

Feed mill biosecurity is important to the feed and animal agricultural industries as a way to control the spread of feed-borne diseases. In the last five years, there has been an urgency to increase feed mill biosecurity measures, especially since the outbreak of Porcine Epidemic Diarrhea Virus (PEDV) in 2013. This virus has caused significant animal and economic losses not only in the United States, but also Europe and Southeast Asia.[2,3] Porcine Epidemic Diarrhea Virus is primarily transmitted by fecal-oral contamination; however, biosecurity measures in feed mills and farms have increased due to the fact that epidemiological evidence linked feed to viral transmission.[4,5,6] Complete feeds and ingredients have been shown to be potential vectors for PEDV.[3,4,5] More recently, mammalian orthoreovirus has also been shown to be present in blood meal, which is an ingredient commonly used in animal feeds, and thus the possibility of feed ingredient transmission of the virus exists.[7]

While little virus transmission through feed was demonstrated prior to 2013, bacterial contamination has been documented for decades. Particularly, *Salmonella* contamination of pet foods has caused a number of recalls in recent years. While *Salmonella* in livestock feeds has not received as much attention in the US, this has been the primary pathogen of concern for hygienic feed production. Still, *Salmonella* may be a pathogen of growing importance in the animal feed industry as consumers and regulators continue to demand more stringent protocols for farm-to-fork food safety. The primary concern with the pathogen is contamination in the feed mill which creates the potential for long-term cross-contamination due to its spore-forming nature. For example, ingredients of animal origin were found to have an 8.8% contamination rate for *Salmonella* in one study, but dust samples collected from the three feed mills documented a contamination rate of 18.5%.[8] The FDA has used surveillance sampling to evaluate the pathogen load in feed ingredients and found contamination rates of 30.9% from 2002 to 2006, and 19.4%

from 2007 to 2009.[9] The contamination rates were much lower in complete feeds, 9.4% and 5.6% at the same respective time intervals, but many of these feeds would still have been considered adulterated by the Food and Drug Administration (FDA).[9] While any serotype of *Salmonella* is considered an adulterant to pet foods, only those pathogenic to animal health are considered adulterants in livestock feed.[9] For example, in swine feed the only zero tolerance serotype is *S. Choleraesuis*.[90] Some feeds will undergo a commercial heat step which is capable of destroying bacteria. However, if the feed is still contaminated after thermal processing, it is still considered adulterated.

These recent outbreaks of PEDV in the swine industry and *Salmonella* recalls in the pet food industry have increased awareness that microbial hazards may be of concern in animal feed manufacturing, and therefore, biosecurity is important to extend to the feed mill. Of course, implementing biosecurity plans in feed mills is challenging because each feed mill is different, and some biosecurity measures that are needed for one facility are not as relevant for another. Regardless of the feed mill, a trained employee, operator, or third party, should first identify the hazards for the mill and assess their relative risk. Next, steps should be taken to control entry or proliferation of those hazards in ingredients and feed upon entry, and any open point of entry into the feed manufacturing system. Once hazards are identified, protocols should be in place to minimize hazards that are inadvertently introduced and to prevent cross-contamination throughout manufacturing. If, at any point, a contaminant does enter the feed mill and causes any issues to the animal health, cleaning of any equipment and recalls of finished product should be evaluated. A well-designed and implemented biosecurity plan for a feed mill increases the protection from microbial pathogens in the farm-to-fork system, which protects potential herd health and economic losses and ultimately minimizes risk of transfer into the foods consumed by

humans.[11] The objective of this review is to identify biological hazards that may be present in animal feed, locations of potential entry of these hazards, and suggested practices for a feed mill biosecurity plan.

Hazards Analysis

Construction of a Flow Diagram

The first step is to first create a block flow diagram to visualize the major manufacturing processes within the feed mill (Figure 1.1). This diagram allows one to easily identify the major processing methods that should be considered in a biosecurity plan for both points of entry and control points. Common categories in the diagram include receiving, processing, storage, packaging, loading, and delivery.[12] A more complex flow with conveying systems can help identify areas that are of higher risk for cross-contamination throughout the manufacturing process. It is also important to point out that every feed mill will have a different flow diagram based on their system.

Hazard Identification

Hazard analysis is the first step in both writing and executing a biosecurity plan, and is getting increased recognition due to Food Safety Modernization Act (FSMA). The Food Safety Modernization Act will release the final rules for animal food on August 31, 2015, followed by the implementation of those rules. The purpose of FSMA is to shift the focus of food safety hazards from responsive to preventive in order to improve food safety in a systems manner.[1] Most feed mills will be required to comply with the Hazard Analysis and Risk-Based Preventive Controls outlined by subpart C of FSMA, which will include written food safety plan that addresses hazard identification, analysis, and risk mitigation.[1] For FSMA, the FDA will be categorizing hazards in a similar manner to the Draft List of Potentially Hazardous Contaminants

in Animal Feeds and Feed Ingredients. Guidance on classification of feed hazards is currently ongoing by the FDA, but feed mills are expected to identify and analyze hazards according to their unique situation.

The FDA Animal Food Safety System developed a Draft List of Potentially Hazardous Contaminants in Animal Feeds and Feed Ingredients in 2006, and this list is a good resource for beginning the hazard identification process.[13] Physical hazards are limited to plastic, glass, metal, bones, and radiation from implanted devices in animals.[13] Chemical hazards include a number of pesticides subject to harmful residues, mycotoxins, heavy metals and radionuclides, and other chemicals such as ethoxyquin, dioxin, polychlorinated byphenyls and selenium.[13] Biological hazards are grouped into two categories: transmissible spongiform encephalopathies, including bovine spongiform encephalopathy and chronic wasting disease, and microbiological contaminants including *Bacillus spp., Clostridium spp., Escherichia coli, Mycobacterium spp., Pseudomonas spp., Salmonella enterica,* and *Staphylococcus spp*.[13] Interestingly, only bacteria were listed as potential microbiological contaminants when the list was created in 2006. With increased knowledge regarding the transmissibility of some viruses by feed, it is important to recognize that one may need to go beyond the scope of this list to identify potential hazards. Reviewing recent literature and other publications, such as the FAO Manual of Good Practices for the Feed Industry, are other good methods to identify hazards that may be present in a facility.

Hazard Evaluation

The second step of hazard analysis is to evaluate the hazard's relative risk to your facility. For example, a feed mill that manufactures feed for both dairy and turkeys may consider *Salmonella Enteritidis* to be a potential significant hazard due to its pathogenicity in poultry,

while one manufacturing feed only for dairy cattle may not consider it a significant hazard that is reasonably foreseeable and therefore unnecessary to control. The FDA has suggested a number of criteria that may be important to evaluate during hazard analysis. These include the formulation of the feed, the type of facility and equipment, raw materials and ingredients, transportation practices, manufacturing and processing procedures, the feed's intended or reasonably foreseeable use, and feed mill housekeeping.[12] Other considerations to determine the relative risk of a hazard include its history of occurrence within the facility, its likelihood of occurrence if a control is not implemented, and the severity of the hazard if it were to occur.

Once one identifies hazards and determines those that are significant and reasonably foreseeable, strategies should be implemented to control those hazards throughout the production process. Certainly these control steps may transcend particular hazard categories, but the emphasis of this review is to create a practical implementation plan for minimizing microbiological contaminants.

Hazard Control

Prevention of Hazard Entry During Ingredient Receiving

One of the most effective methods to address feed mill biosecurity is to prevent hazard entry during the receiving of ingredients.[12] The European Food Safety Authority (EFSA), has shown that the introduction of a contaminated material in a feed mill can lead to the feed mill being contaminated for an extended time under certain conditions.[14] The first step for hazard prevention during receiving is to develop a supplier verification program that includes purchase specifications that clearly communicate your expectations for the safety of inbound ingredients. Also, this may include verification of ingredients supplier protocols and on-site manufacturing facility reviews and assessments. Once those specifications are in place, it is important to enforce

them. A strict analytical schedule should be created and enforced for particular pathogens within high risk ingredients. This analytical schedule will be dependent upon each feed mills high risk pathogens and ingredients. This schedule should also include testing and holding procedures. In addition, sampling protocols should be constructed that identify sampling method, quantity needed to collect, sample labeling and retention procedures, and the directions for analysis.[12] The Association of American Feed Control Officials' Feed Inspector's Manual (5th ed.) outlines aseptic sampling methods to obtain a high quality representative sample from various types of ingredients.[15,16]

The receiving process is also an area where emphasis can be placed on requirements for inbound trucks. Recommendations for feed mills producing feed for swine breeding stock production in the PIC USA system suggest all trucks entering the feed mill should have mud and sludge removed from the trailer opening before the truck reaches the pit, and the pit should remain covered until the truck is ready to unload.[17] Cones and funneling devices can also be used to limit the amount of material that misses the pit and help prevent people from sweeping spilled ingredients into the pit.[17] Material should never be swept from the floor or off of the trucks into the pit. If any material is spilled or falls off the truck, it should be disposed of and not placed back into the system.

Directions for the truck drivers should also be posted on proper signage (figure 1.2) in order for them to be able to follow the appropriate security measures.[12] Ideally, drivers should stay inside trucks at all times to minimize foot traffic. If the driver needs to leave his truck, he should wear disposable plastic boots or use foot baths to prevent pathogen entry.[17]

In addition, covers should be placed over the pit area until a truck is ready to unload to help prevent foreign material from entering the pit itself and to limit pest entry (figure 1.3).

Required documentation, such as receiving records that include the date, time, and lot number during unloading, should also be gathered in order to improve traceability if an outbreak occurs which allows the feed and ingredients to be traceable. Documentation from the inbound truck should also be collected on what was hauled in the truck prior to the ingredient that is being unloaded. Regardless if ingredients enter the feed mill in bagged, bulk, or liquid form, particular emphasis should be placed on sampling and pathogen analysis of high risk ingredients prior to unloading. Particularly, bulk ingredients typically enter through a central pit and travel through bucket elevators, turn heads, and conveyors to storage bins. Ingredients may be contaminated prior to unloading, but they may also be contaminated during the unloading process due to mud or floor sweepings intermingling with ingredients in the pit. Historically, there has been little emphasis on the unloading and sequencing of high risk ingredients or the disposal of floor sweepings in other locations, but these practices should be considered to reduce the risk of microbial contamination in inbound ingredients.[14] Also, the receiving pit and the conveying equipment from the pit (figure 1.4-1.6) is not practical to clean on a frequent basis and may have considerable ingredient excess left in them. Therefore, the potential exists for cross-contamination of hazards from one source to another. Thus, prevention of pathogen entry into this equipment is critical for prevention of subsequent components of the feed production system.

Bagged ingredients are typically stored in their original bags within the warehouse until used, while liquids are unloaded into a storage tank that may or may not be heated. Bagged ingredients should be checked to ensure that bags are intact and dry. Lot numbers should be recorded and samples collected for microbiological analysis. Liquid ingredient valves should

also be locked when not in use. If the ingredient is heated, steps should be taken to ensure proper heating to prevent microbial growth in the water fraction of the liquid.

Prevention of Hazard Entry Due to People

One of the most overlooked areas of hazard entry is from people, both those working in the feed mill and visitors like guests, truck drivers, and subcontractors. Some of the most common breaches in biosecurity occur when visitors, like subcontractors, enter the facility. People may unknowingly carry fecal, dirt, or dust particles contaminated with microbial pathogens on the bottom of their shoes or clothing, and are at a particularly higher risk if they are coming from another farm or feed mill.[15] People movement considerations for biosecurity purposes on swine farms were refined by those researching methods to reduce the transmission of Porcine Reproduction and Respiratory Syndrome(PRRS), but these procedures were never extended to the feed mill because that virus is not known to be transmissible by feed.[18,19] The research on PRRS just goes to show that infection can occur from viral particles on boots, coveralls, and the human body in general. [18,19] However, considering other feedborne pathogens, feed mills may need to consider protocols for personnel movement throughout a system and even within a feed mill. A prime example is the foot traffic of employees and truck drivers that enter a feedmill. As seen with PRRS, they can carry viral or bacterial contaminants on themselves or clothing and introduce it into the system through areas such as receiving grates and hand add areas. Especially truck drivers who are delivery feed to farm areas. Another potential vector to take into account is if an ingredient is potentially contaminated, employees could walk through the ingredients or dust and potentially spread it throughout the feed mill. To better understand the magnitude of the potential risks of foot traffic, recent research with PEDV can again be used as an example. Based on the minimum infectious dose of PEDV in a swine diet, the magnitude

of infectivity of PEDV was calculated to be the same as one gram of PEDV-infectious feces blended into 500 tons of feed with all the resulting feed being capable of creating infectivity.[20]

Log books should be available to document the entry and exit time of visitors.[12] Procedures should outline that visitors must be accompanied at all times by a trained employee to help prevent biosecurity breaches. Visitors should wear plastic boots or use footbaths to limit the entry of outside pathogens.[12] Finally, signage should be created in appropriate areas to communicate any off-limit areas.[12]

Prevention of Cross-Contamination of Hazards During Production

If biosecurity measures fail and microbial pathogens enter the facility, it is very difficult to remove them from the system.[14] Cross-contamination is the contamination of a adulterant-free product by contaminated feed or ingredients within the production process. Any location where there is the propensity for residual organic matter to remain after processing within equipment can lead to potential cross-contamination of subsequent batches or runs. The highest risk for this to occur may include screw conveyors, boot pits of bucket elevators, coolers, and storage bins. Sequencing of high-risk ingredients and feeds from receiving through load-out and delivery may reduce the risk of cross-contamination of microbiological pathogens, but preliminary data from our laboratory indicates that infective PEDV is still present in the second feed batch sequenced after the manufacturing of a contaminated batch.

Secondary to prevention is limiting microbiological hazards in the manufacturing area by the use of a strict housekeeping schedule.[12,17] This schedule can include sweeping production areas such as the floors and hand-add areas on a regular basis and disposal of the sweepings into the trash. Particular emphasis on housekeeping should occur in high traffic areas and locations with feed contact surfaces. An important part of housekeeping is dust collection. Notably, many

feed mills place dust from the air collection systems and floor sweepings directly back into the feed system to limit shrink. However, this dust should be considered as high risk and thus disposed.[14] Flushing and sequencing schedules to minimize cross-contamination after manufacturing high risk feeds should be well-defined in a biosecurity plan for clarity. Flushing is "The process of running an ingredient through the manufacturing equipment and associated handling equipment after the production of a batch of feed, for the purpose of cleaning out any drug residue."[21] Sequencing is "The preplanned order of production, storage, and distribution of different animal feeds designed to direct drug carryover into subsequent feeds which will not result in unsafe contamination."[22] Both a flush and sequence may be considered if using a particular high risk ingredient.

Prevention of Cross-Contamination of Hazards During Load-Out and Delivery

Similar to receiving, the control of cleanliness of outbound trucks is important to ensure feed safety (Figure 1.7). The exterior, top, and interior compartments of trucks should be inspected for cleanliness prior to entering the load-out bay or loading of ingredients. Documentation that includes the previous load hauled, shipment lot number and location, and time of loading should be maintained to improve traceability.[12] Many times trucks are sequenced or designated for specific sites so feed is delivered to low risk sites prior to higher risk sites.[17] Some facilities have incorporated truck washes, bakes, and sanitation methods for feed trucks to minimize the contamination risk of the feed mill, feed, and other farm sites.[17]

A biosecurity plan should also include specific directions for drivers during delivery. For example, truck drivers would ideally stay in their trucks during delivery and an on-site worker would open any bin lids. Contaminated feed bins on farms can lead to the infection of animals

which is why it is important for the driver to not come into contact with the bin.[4] This is still relatively impractical for most sites, so if a driver needs to exit a vehicle, they should wear plastic boots and sanitize their hands prior to entering their truck again.[12,17]

Mitigation of Hazards

Beyond prevention, proactive mitigation helps to reduce the risk of microbiological pathogens in complete feed and is therefore an important consideration when creating a biosecurity plan. For example, thermal processing by pelleting has been demonstrated to significantly reduce the quantity of both PEDV and *Salmonella* when processing at manufacturing temperatures.[20,23] While it is not a stand-alone kill step for bacterial pathogens, a pellet mill may help reduce the risk of microbial hazards. Still, one must remember that pelleting is a point-in-time mitigation step that does not prevent subsequent recontamination, and pelleted feeds can still contain these biological contaminants upon final delivery.[23] This can occur in areas such as the cooling process since air is recycled through the feed mill. The external application of chemicals like organic acids or formaldehyde may be an attractive risk mitigation step because the chemicals carry residual activity that can prevent recontamination.[24,25] Formaldehyde is approved for the mitigation of *Salmonella* in animal feed, but proper application requires specific equipment and the chemical may carry a negative connotation for consumer and worker safety risks. Some chemical alternatives, such as medium chain fatty acids or essential oils, show promise as microbial contaminant mitigants and are just as effective as formaldehyde, but current tested levels are uneconomical and impractical for implementation.[24,25] Further research is important to evaluate the value of more practical inclusion levels of these feed additives.

In summary, prevention of the hazard entry is the first priority of a biosecurity plan. However, an effective plan should also address methods to reduce cross-contamination or to

mitigate the pathogen if it enters the facility. A holistic approach to feed mill biosecurity is necessary to maximize risk reduction of microbial hazards.

Assessments

The final step of a biosecurity plan should be an assessment plan to evaluate the effectiveness of procedure implementation and expose areas of risk that need to be addressed.[12,18] It may be helpful to construct a self-assessment with simple 'Yes' and 'No' answers addressing the points that were outlined above. Proactive assessments are most useful if conducted between every 3 and 12 months.[18] During this time, the assessor should document areas where the biosecurity plan should be changed due to impracticality or to increase effectiveness. It is important to point out that under FSMA there is not a requirement for a 3rd party assessment, but it could be beneficial to have an assessment done by a 3rd party who is educated in biosecurity measures.

Conclusions and Future Approaches

The emphasis on feed mill biosecurity has increased due to research demonstrating that feed can be a vector for pathogens like PEDV and *Salmonella*. A biosecurity plan requires the identification and evaluation of hazards, as well as control procedures for significant hazards that are reasonably foreseeable to occur within a particular facility. These control procedures can include the prevention of entry of the pathogen during receiving or by people, prevention of cross-contamination of hazards, and proactive mitigation using thermal processing or chemical additives. Many of these strategies are included in good manufacturing practices and quality control programs, but a separate biosecurity plan may be helpful to concentrate efforts. An assessment strategy, such as a self-assessment, may help facilities identify gaps in their biosecurity plans. Future research is needed to continue to quantify the relative risk of pathogens

in various feeds and ingredients to particular species and to elucidate improved control methods. Still, constructing a biosecurity plan is important because extending biosecurity from the farm to the feed mill will reduce the risk of microbiological hazards in feed and therefore improve herd health, economic security, and farm-to-fork food safety.

It is important to point out that this review was written with a systematic approach to solving biosecurity issues in a feed mill for the swine industry. This review lists recommendations to improve biosecurity in a feed mill and should not be viewed as a list of requirements; however, using some of these suggestions could increase not only the biosecurity of the feed mill but also herd health, economic losses, and ultimately minimize risks of transfer into the foods consumed by humans. It is also important to understand that the implementation of biosecurity procedures will also come with associated manufacturing costs and, in turn, an increase in the feed costs for the mill consumer. In some instances it may also require a feed mill to hire an extra employee to help with unloading and sampling procedures. With the implementation of the biosecurity measures, other auctions will need to be taken such s proper training to prevent slips, trips, and falls when people are using plastic booties and foot baths. However, with the ultimate goal of producing a safe and nutritious food source for the animal the feed mills serve, the benefits of a biosecurity plan greatly outweigh any negative consequences.

Summary of Suggested Practices and Key Points

The list below is a summary of the recommendations for high biosecurity and key points. It is important to note that the implementation of these biosecurity measures will have certain cost associated with them. Feed mills can use all or some of the recommendations to increase their biosecurity based on their level of risk.

1. Establish supplier verification which includes purchase specifications and safety of inbound ingredients.
2. Inbound trucks should have all the mud and sludge removed from the trailer opening.
3. Signage should be placed with the security measures for drivers to follow.
4. Covers should remain over the dump pit until the truck is ready to unload
5. Contamination can occur from workers, guests, drivers, and subcontractors.
6. Pathogens can be carried on clothing and footwear
7. Visitors, drivers, and subcontractors should wear plastic booties, or use footbaths.
8. Signage should be used to show off limit areas to unauthorized personnel.
9. Dust is capable of carrying pathogens.
10. Use a strict housekeeping schedule which includes cleaning out available equipment and sweeping floors.
11. Any dust collected should be disposed and not placed back into the manufacturing system.

12. In order to clean out equipment that cannot be opened, a flush or sequence can be used. If a flush it used it should be disposed of.

13. Outbound trucks should be inspected for cleanliness prior to entering the load-out area.

14. The use of farm specific trucks should be incorporated if possible. This allows for one truck to only be dedicated to one farm site.

15. Drivers should be given specific directions for delivery which includes how to enter and exit farms and unload feed.

16. If possible an onsite worker should open bins for the driver si that the driver does not have to exit the vehicle.

17. If the driver does exit the truck they should wear plastic booties and then sanitize their hands prior to entering the truck.

18. An assessment should be carried out which can consist of a self-audit.

19. A self-audit should be conducted between every 3-12 months depending on the risks of the feed mill.

20. A third party audit could be beneficial to help find biosecurity issues in the feed mill.

21. Link for FSMA-

    http://www.fda.gov/Food/GuidanceRegulation/FSMA/

22. Link for Sampling Video-

    https://www.youtube.com/watch?v=dX6BLn9WKGE&feature=youtu.be

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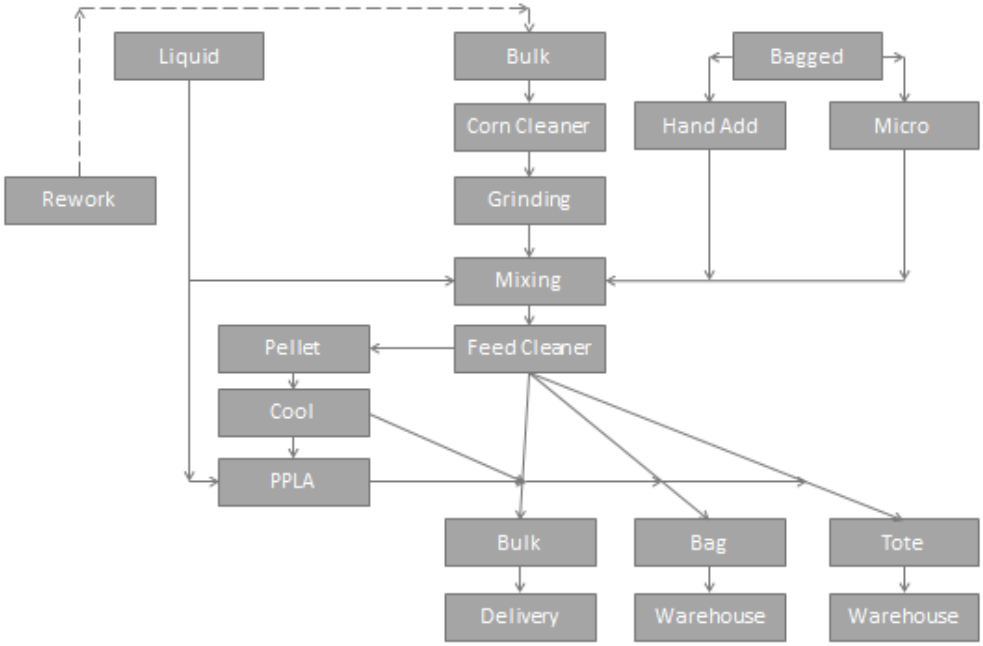
Figures and Tables

Figure 1.1. Block flow diagram of a feed manufacturing process



[1] Creating a flow diagram of a facility is an easy way to visualize which processes must be considered in the biosecurity plan. A more complex flow that includes conveying equipment may help isolate locations where cross-contamination is of higher risk to occur.

Figure 1.2 Example of potential contamination entering the dump pit by truck

Figure 1.3 Example biosecurity sign with directions for truck drivers

Figure 1.4 Screw conveyor with potential contamination

Figure 1.5 Feeder with potential contamination

Figure 1.6 Bucket elevator with potential contamination

Figure 1.7 Potential contamination from left over material in the top of a bulk feed truck

Chapter 2 - Evaluating Chemical Mitigation of Porcine Epidemic Diarrhea Virus (PEDV) in Swine Feed and Ingredients

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Abstract

Porcine Epidemic DiarrheaVvirus (PEDV) is transmitted by fecal-oral contamination. Research has confirmed swine feed or ingredients as potential vectors of transmission, so strategies are needed to mitigate PEDV in feed. The objective of this experiment was to evaluate the effectiveness of various chemical additives to prevent or mitigate PEDV in swine feed and ingredients that had been contaminated post-processing. Treatments were arranged in a 7 × 4 factorial with 7 chemical treatments and 4 feed matrices. The chemical treatments included: negative control with no chemical addition, 0.3% commercial formaldehyde product, 1% sodium bisulfate, 1% sodium chlorate, 3% custom organic acid blend (OA), 2% custom essential oil blend (EO), and 2% custom medium chain fatty acid blend (MCFA). The 4 matrices included a complete swine diet, blood meal, meat and bone meal, and spray-dried animal plasma. Matrices were first chemically treated, then inoculated with PEDV, stored at room temperature, and analyzed by RT-PCR on d 0, 1, 3, 7, 14, 21, and 42. Formaldehyde, MCFA, EO, and OA addition each decreased RNA concentration of PEDV compared to the control ($P < 0.05$), with formaldehyde being the most effective on d 0. Feed matrix appears important in PEDV detection as RNA concentrations were greater in the swine diet and blood meal than meat and bone meal or spray-dried animal plasma on d 0 ($P < 0.05$). Additionally, PEDV stability over time was influenced by matrix as RNA concentrations were greater by d 42 for spray-dried animal plasma and meat and bone meal than the complete swine diet and blood meal. 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. Notably, the MCFA was equally as successful at mitigating PEDV as a commercially-available formaldehyde product.

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 coronavirus affects pigs of all life stages, but the highest mortality rates are seen within sucking pigs because of their less developed digestive tracts [3]. The virus is known to 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]. Viral transmission by feed may be by direct contamination, but is more likely from cross-contamination during the manufacturing, transportation, and storage of feed and ingredients [4].Viral destruction by thermal processing or irradiation is important to evaluate, but both are point-in-time mitigants that do not offer residual protection from contamination post-processing, which is a solution offered by chemical treatment. Chemical additives, such as formaldehyde, have been demonstrated to be effective at mitigating *Salmonella* in animal feed, and research suggests it may be effective in PEDV destruction [5, 6]. However, formaldehyde does not have regulatory approval for PEDV mitigation in the United States, requires specialized equipment for successful application, carries potential worker health concerns, and may be perceived negatively by consumers [7, 8]. Alternatively, chemical additives may provide solutions to these issues. Medium chain fatty acids have shown to be effective at mitigating enveloped viruses and bacteria, but the concentrations that are required to inactivate the virus can be upward of 20-fold the normal application levels [9, 10]. Organic acids have been studied as an antimicrobial agent for several decades, and have shown to be effective at bacterial mitigation and some extremely detrimental viruses, such foot and mouth disease and African swine fever [10, 11, 12]. However, the knowledge of effectiveness of organic acids against other viruses, such as PEDV, is somewhat limited. Essential oils have also showed antimicrobial effects, as well as antiviral

RNA effects [13]. Sodium bisulfate is a commercial product, which is used in the broiler and pet food industry for microbial control, particularly against *Salmonella*. 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 because its dry powder form may be more easily implemented by the feed industry compared to other liquid chemicals [14]. Finally, sodium chlorate has shown to be effective at pathogen mitigation when included in drinking water of livestock [15] and for PEDV mitigation of surfaces [16]. Because of various physical states, chemical composition, and electrostatic properties of each chemical additive and feed matrix, each additive may interact differently as a mitigant. Therefore, the objective of this experiment was to evaluate the effectiveness of various chemicals to mitigate post-processing PEDV contamination in swine feed and feed ingredients.

Materials and Methods

Chemical Treatment

Seven chemical treatments were applied to four different feed matrices. The chemical treatments included: 1) negative control with no chemical addition, 2) 0.3% commercial formaldehyde product (Termin-8, Anitox Corp, Lawrenceville, GA), 3) 1% sodium bisulfate (Jones-Hamilton Co, Walbridge, OH), 4) 1% sodium chlorate, 5) 3% OA blend [1:1 ratio of lactic, propionic, formic, and benzoic], 6) 2% essential oil blend [1:1 ratio of garlic oleoresin, turmeric oleoresin, capsicum oleoresin, rosemary extract, and wild oregano essential oils], and 7) 2% medium chain fatty acid blend [1:1 ratio of caproic, caprylic, and capric acids]. The 4 matrices included: 1) complete swine diet, 2) blood meal, 3) porcine meat and bone meal, and 4) spray-dried porcine plasma. None of the matrices had previous chemical mitigants added and

were tested for proximate analysis (Tables 2.1). The complete swine diet was a Phase 3 swine nursery diet manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center. All protein meals were obtained in dried form and untreated with preservatives, antimicrobials, or other chemicals. 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-PCR prior to chemical treatment. One kilogram (kg) of each feed matrices was placed in a lab scale ribbon mixer where the liquid chemicals were fogged onto the feed and the powered treatments were mixed directly into the mixer. All chemical 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 was collected from 10 different locations and placed into a polyethylene container for inoculation. Between protein meals of the same chemical treatment, the mixer was physically cleaned to remove all organic residue. Between different chemical treatments, the mixer was physically and wet cleaned and dried to remove all organic and chemical residue. A ground corn flush between treatments was also used to prevent treatment-to-treatment cross-contamination.

Inoculation

The 28 samples were inoculated with PEDV in polyethylene containers at the Kansas State University Veterinarian Diagnostic Lab using USA/IN/2013/19338 Passage 7 grown in Vero cells with an infectious titer of 5.6 x 10^5 TCID50/ml. A total of 10 mL (1 ml cell fluid + 9 ml cell culture fluid) 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 matrices were divided into twenty-one 3-g sub-samples and placed into 15 mL conical tubes. Tubes were stored at room temperature until analyzed by RT-PCR. There were three replicates per sub-sample. Untreated control 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 analysis to determine intra- and inter-assay variation. There was very little variation among sampling day or within duplicate, suggesting that the RT-PCR assay was highly sensitive, accurate, and precise (Table 2.2).

Real-Time PCR Analysis

Twelve milliliters (mL) of 1× PBS (Life Technologies, Grand Island, NY) was added to each three gram 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 quantitative reverse-transcription PCR (qRT-PCR). Analyzed values represent threshold cycle (CT) at which the virus was detected, and thus lower values indicate greater nucleic acid presence, not infectivity.

A duplex qRT-PCR 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-PCR kit (Life Technologies, Grand Island, NY) in a 20 µL reaction. qRT-PCR 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 qRT-PCR 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.

Results

All main effects and interactions were highly significant ($P < 0.001$; Table 2.3). Overall, the commercial formaldehyde product, MCFA, EO, OA, and sodium chlorate all differed from the control ($P < 0.05$). The commercial formaldehyde was the most effective chemical treatment overall (32.5 CT), followed by the MCFA (31.4 CT) EO (30.5 CT), and OA treatments (30.4 CT), all of which improved ($P < 0.05$) the quantity of detectible PEDV nucleic acid compared to the untreated control as detected by RT-PCR (Table 2.4).

Significant differences were also observed between each of the feed matrixes ($P < 0.05$). Overall, blood meal had the highest PEDV CT (32.9 CT), followed by the complete swine diet, spray-dried porcine plasma, and porcine meat and bone meal ($P < 0.05$; 32.0, 29.2, and 28.1 CT, respectively; Table 2.5).

Time also affected PEDV concentration detected by RT-PCR, with d 0 and 1 being statistically similar (29.0 vs. 28.8 CT, respectively; $P > 0.05$), but lower ($P < 0.05$) than d 3 (29.8 CT; Table 2.6). The CT increased over time during d 3, 7, 14, and 21 ($P < 0.05$; 29.8, 30.6, 31.1, and 32. 1, respectively). However, d 21 and 42 were similar ($P > 0.05$) overall (32.1 vs. 32.3 CT, respectively).

Interactions are presented graphically and provide more relevant results regarding the effects of specific chemical mitigants in various matrices over time. The PEDV CT in the untreated control of the complete swine diet increased until d 21, after which it remained relative constant (Figure 2.1). Of the tested chemical 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 (Figure 2. 2). Although the EO treatment was not effective at mitigating PEDV according to RT-PCR through d 7, it was the most effective on d 14, 21, and 42.

Interestingly, the PEDV CT in the untreated control of the porcine meat and bone meal was highly stable throughout the experimental period, with no chemical showing substantial mitigative effects, even though differences were statistically significant (Figure 2. 3).

The PEDV CT in the untreated control of the spray-dried porcine plasma was also relatively stable over time (Figure 2. 4). However, the commercial formaldehyde product was highly successful at mitigating PEDV according to RT-PCR in spray-dried porcine plasma compared to other tested chemical additives.

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-PCR (Figure 2. 5). Again, the PEDV CT in blood meal and complete swine diet increased over time consistently until d 21, but were relatively stable from d 21 to 42. Meanwhile, the porcine meat and bone meal and spray-dried porcine plasma maintained the PEDV CT more consistently over time.

Discussion

The purpose of this experiment was to evaluate possible chemical treatments as PEDV mitigation strategies by the use of RT-PCR analysis. Clearly, its primary limitation is that PEDV infectivity was not confirmed by swine bioassay. Still, this research is relevant because evaluating the RNA concentration by RT-PCR is currently the most practical method to assess the PEDV risk in swine feed or ingredients. Surprisingly, the PEDV concentration was relatively stable in spray-dried porcine plasma and porcine meat and bone meal, while the RNA detected substantially declined during the initial 21-d period in the complete swine diet and blood meal. Similar findings have been previously reported, where temperature, relative humidity, and the storage environment have had varying effectiveness on PEDV mitigation [17]. Under the laboratory conditions, PEDV was successfully mitigated in different feed matrices by a commercial formaldehyde product, MCFA, OA, and EO. Interestingly, the most effective mitigants were liquids compared to dry powders, suggesting that physical properties of chemical

mitigants are important to consider. Others have demonstrated the effectiveness of OA on PEDV mitigation, with different combinations of OA showing varying inactivation kinetics [18].

The commercial formaldehyde product in this study performed similarly in complete feed as was observed by Dee et al. [6]. Our research demonstrated a more consistent degradation curve comparatively, but always maintained a PCR concentration below the 40-CT threshold, whereas Dee et al. [6] found formaldehyde treatment resulted in PEDV non - detectable readings (> 40 CT) on d 7 and 13, but not d 9, 11, or 15. These differences may be attributed to varying commercial products as this study used Termin-8 and the study by Dee used Sal CURB. [6] Another difference is in the application techniques. In the present study, a pilot-scale laboratory ribbon mixer fitted with aerosolizing equipment was used to mimic the commercial chemical application process, and the mixer had a coefficient of mixing variation less than 7%. Alternatively, Dee et al. [6] utilized more simplistic mixing equipment that may have resulted in less efficient chemical distribution.

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 [12]. Porcine Epidemic Diarrhea Virus can be related to classical swine fever since they both are positive sense RNA viruses. Based on this 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, such as formaldehyde [5, 19]. One of the major differences is that this study used 0.003% formaldehyde compared to 1 to 2% for the avian influenza virus [19]. If 1 to 2% of the formaldehyde product would have been used, then it is possible that there could have been a higher sensitivity of PEDV to the treatment. It has been pointed out that the presence of lipids in a virus and the size of the virus could be two factors that influence the mode of action of

the chemicals [20]. This could be one of the main reasons the virus can be sensitive to the addition of formaldehyde and other lipid solvents.

Essential oils have also 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 into the host cells [21]. With that said, it is possible that the EO used in this study could be masking certain components of the virus causing the disruption of the envelope.

Medium chain fatty acids such as capric acid have shown to be effective against some RNA viruses such as visna virus and vesicular stomatitis virus [22]. Capric acid was also used in this study as one of the three MCFA tested in combination with two others. It is possible that the medium chain mixture is disrupting the viral envelope as shown by electron microscopy [22].

In this study, it was also observed that PEDV interacts differently within each of the feed matrices. This can also be observed when the virus is thermally treated in different feed matrices [23]. It has also been shown that PEDV survival can be affected by the temperature and relative humidity [23]. This could be one of the reasons that virus stability changed depending upon ingredient or feed matrix.

Conclusions

This is the first research of its kind to evaluate chemical mitigation of PEDV in swine feed and ingredients, and provides valuable information to control the virus by preventing post-processing cross-contamination.

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 viral nucleic acid degrades substantially 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. Further research is needed to correlate findings with infectivity, confirm chemical treatment responses, and refine treatments for both effectiveness and applicability to the swine industry.

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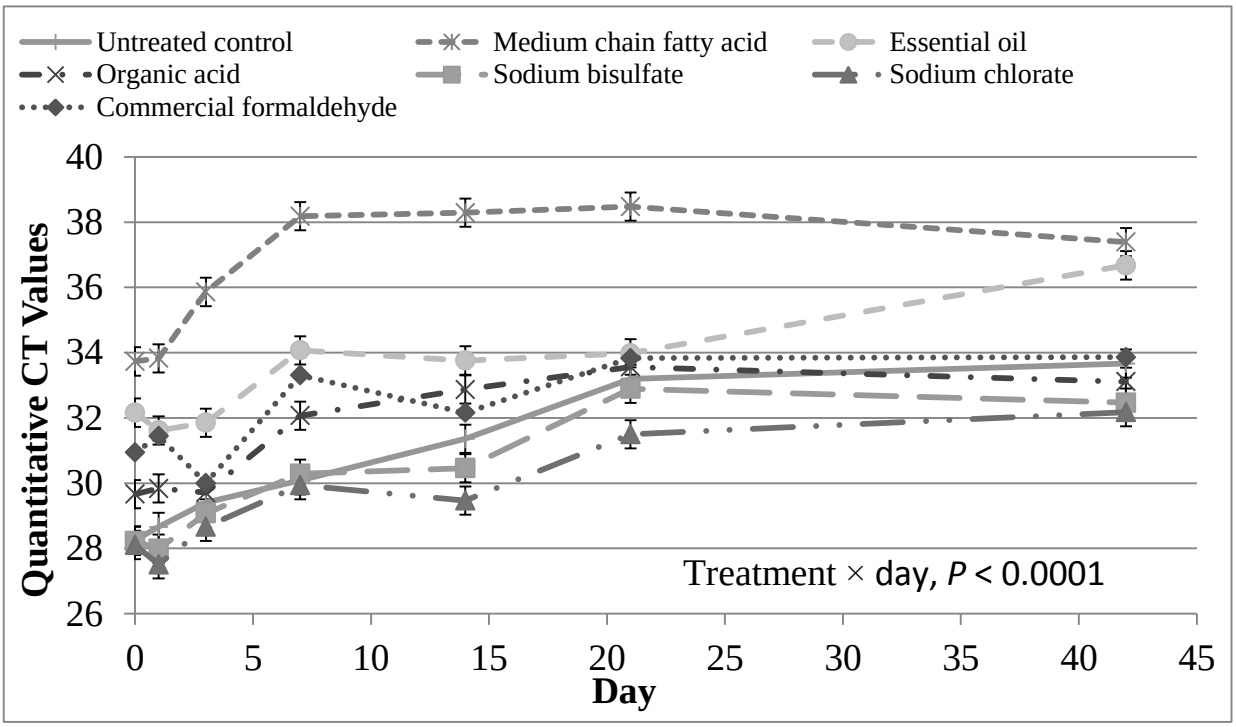
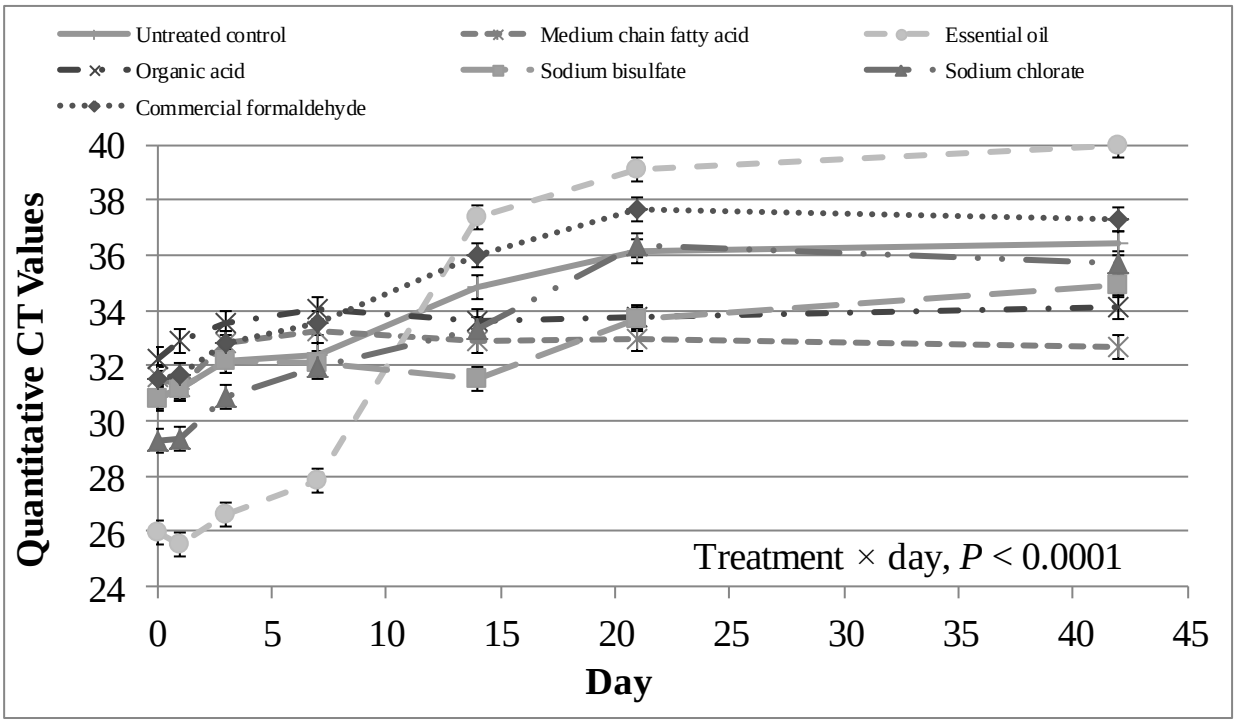
Figures and Tables

Figure 2.1 PEDV contamination post-treatment in complete swine diet stored at room temperature



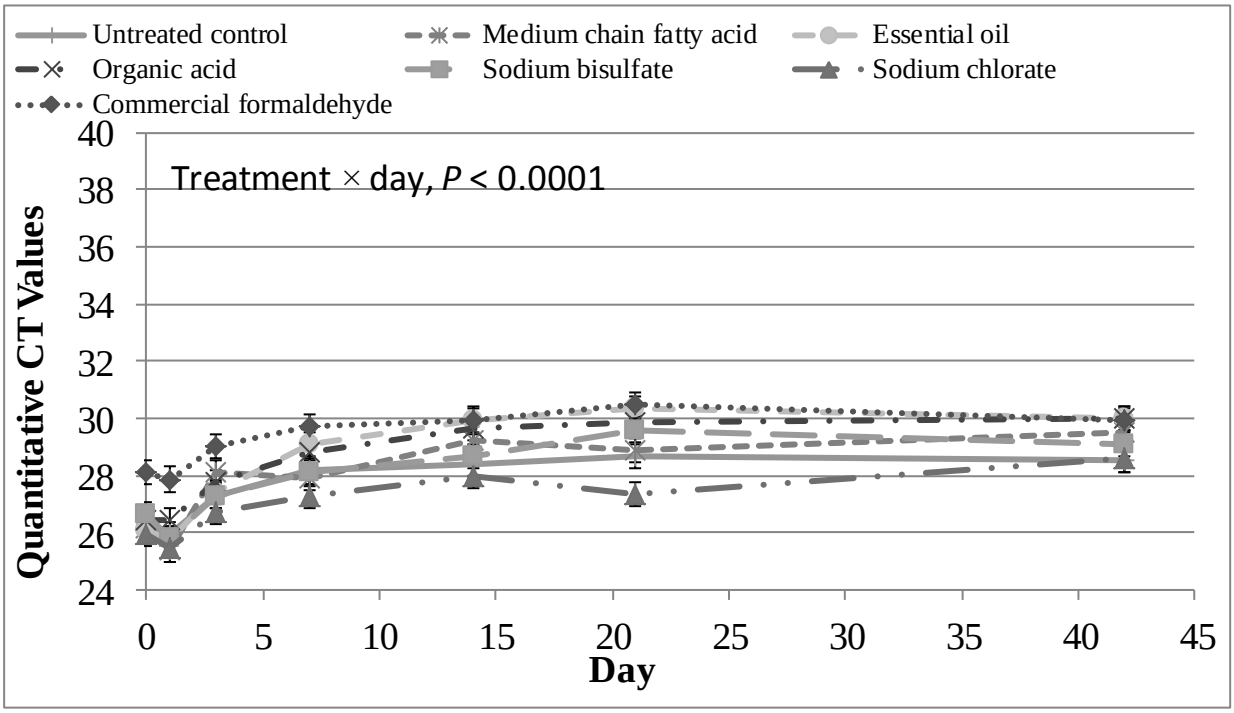
[1]Data was analyzed by PCR with each data point represented by N=3
[2]The higher the CT value, the less quantity of PEDV RNA genetic material is detected.

Figure 2.2 PEDV contamination post-treatment in blood meal stored at room temperature



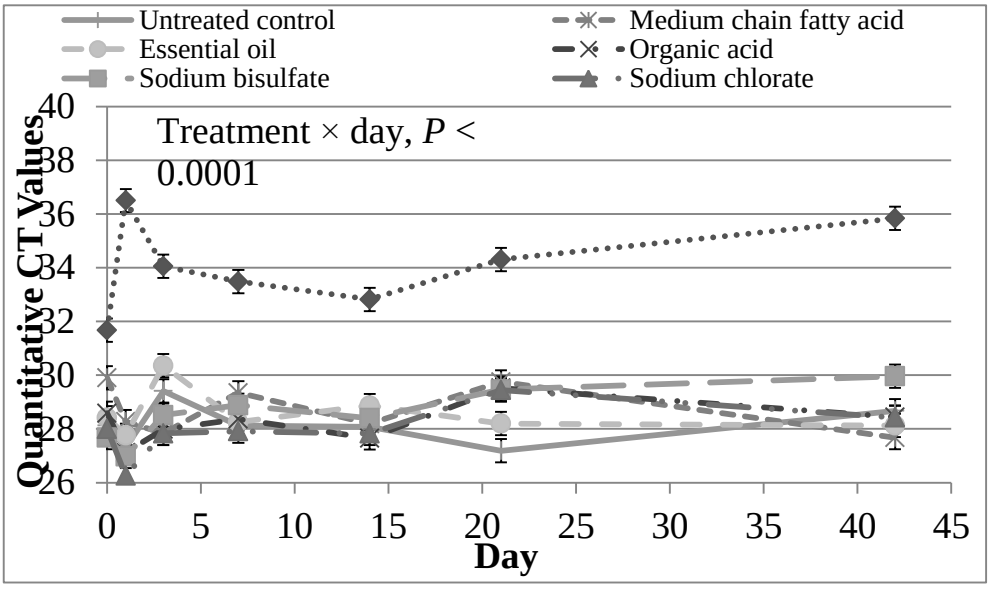
[1]Data was analyzed by PCR with each data point represented by N=3
[2]The higher the CT value, the less quantity of PEDV RNA genetic material is detected.

Figure 2.3 PEDV contamination post-treatment in porcine meat and bone meal stored at room temperature



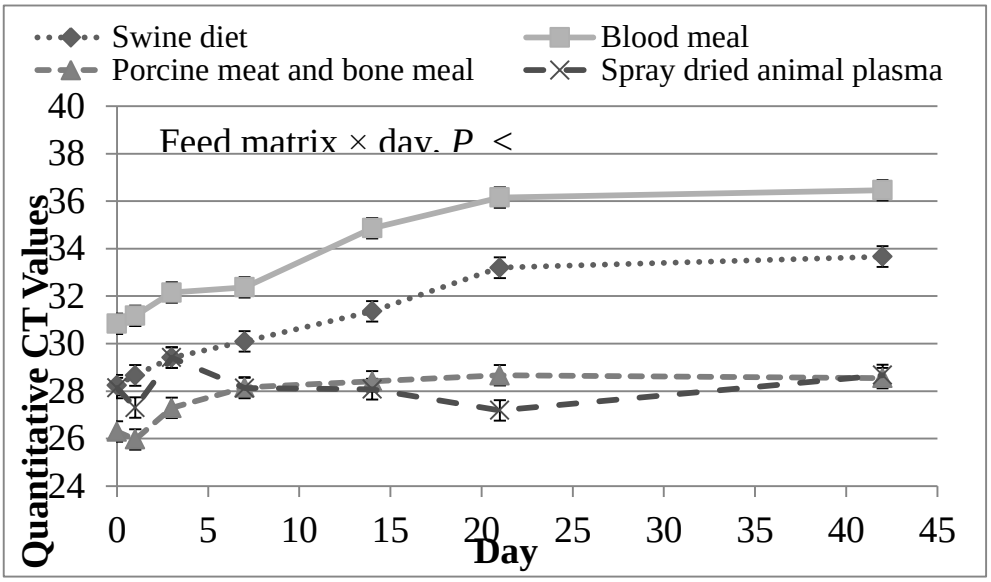
[1]Data was analyzed by PCR with each data point represented by N=3
[2]The higher the CT value, the less quantity of PEDV RNA genetic material is detected.

Figure 2.4 PEDV contamination post-treatment in spray dried animal plasma stored at room temperature



[1]Data was analyzed by PCR with each data point represented by N=3
[2]The higher the CT value, the less quantity of PEDV RNA genetic material is detected.

Figure 2.5 PEDV contamination post-treatment for the untreated controls



[1]Data was analyzed by PCR with each data point represented by N=3
[2]The higher the CT value, the less quantity of PEDV RNA genetic material is detected.

Table 2.1 Proximate analysis of feed matrix (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
Crude protein	21.59	87.17	56.42	79.66
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 Within-day laboratory controls of PEDV-contaminated samples to evaluate the interassay variation

	Day												
Item[1,2]	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. A higher CT value means less genetic material present.
[2]Samples were analysed via real time PCR. On day 0 mean is represented by N=1. On days 1 to 42 means are represented by N=2.

Table 2.3 Main effects and interaction on PEDV quantity as detected by RT-PCR

Effect	*P* =
Treatment	< 0.001
Feed matrix	< 0.001
Day	< 0.001
Treatment × Feed matrix	< 0.001
Treatment × Day	< 0.001
Feed matrix × Day	< 0.001
Treatment × Feed matrix × Day	< 0.001

Table 2.4 Main effects for chemical means for chemically treated PEDV inoculated feed matrices

Item[1]	Control	Essential oil	Medium chain fatty acids	Organic acids	Sodium bisulfate	Sodium chlorate	Termin-8	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 588 samples were used for the analysis. For each treatment means are represented by N=84.
[2] Cycle time required to detect the genetic material. A higher CT value means less genetic material present.
[abcde] Means within a row lacking a common superscript differ $P < 0.05$.

Table 2.5 Main effects of feed matrix on PEDV detection

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 588 samples were used for the analysis. For each treatment means are represented by N=147.
[2] Cycle time required to detect the genetic material. A higher CT value means less genetic material present.
[abcd] Means within a row lacking a common superscript differ.

Table 2.6 Main effects of day of sampling post PEDV inoculation on PEDV detection

	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 588 samples were used for the analysis. For each treatment means are represented by N=21.

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

[abcde] Means within a row lacking a common superscript differ.

Chapter 3 - Evaluating Chemical Mitigation of *Salmonella* in Feed Ingredients

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Abstract

Salmonella is a potential feed safety hazard in animal feed ingredients. Thermal mitigation of *Salmonella* in the rendering process is effective, but it does not eliminate the potential for cross-contamination within rendered ingredients. Therefore, the objective of this experiment was to evaluate the effectiveness of chemicals to mitigate *Salmonella* cross-contamination in rendered proteins over time. Treatments were arranged in a 6 × 4 factorial with 6 chemical treatments: 1) negative control without chemical treatment, 2) 0.3% commercial formaldehyde, 3) 2% essential oil blend, 4) 2% medium chain fatty acid blend, 5) 3% organic acid blend, and 6) 1% sodium bisulfate, and 4 rendered protein meals: 1) feather meal, 2) avian blood meal, 3) porcine meat and bone meal, and 4) poultry by-product meal. Matrices were first chemically treated, then inoculated with *Salmonella* enterica subsp. enterica serovar Typhimurium (ATCC 14028), stored at room temperature, and enumerated via plate counts on d 0, 1, 3, 7, 14, 21, and 42 post-inoculation. Data were analyzed by the GLIMMIX procedure of SAS with day as a repeated measure. The analyzed values represent colony forming units per gram (CFU/g). All main effects and interactions were significant ($P < 0.05$). The *Salmonella* concentration in ingredients treated with medium chain fatty acid and commercial formaldehyde were similar to one another ($P=0.23$), but two logs lower than the control ($P < 0.05$; 0.51 and

0.65 vs. 2.56 CFU/g, respectively). Ingredients treated with organic acid and essential oil treatments also had less *Salmonella* than the control ($P < 0.05$; 1.20 and 2.10 CFU/g, respectively). Time also played a significant role in *Salmonella* mitigation as all days ($P < 0.05$) except d 14 and 21 ($P = 0.92$) were different from one another (4.50, 2.65, 1.75, 0.95, 0.49, 0.50, 0.13 CFU/g for d 0, 1, 3, 7, 14, 21, and 42, respectively). Rendered protein matrix also affected *Salmonella* stability as concentrations in meat and bone meal and blood meal and were similar to one another ($P = 0.36$; 1.82 and 1.73 CFU/g, respectively) but greater than ($P < 0.05$) levels in feather meal and poultry by-product meal (1.36 and 1.36 CFU/g, respectively). In summary, chemical treatment and time both mitigated *Salmonella,* but their effectiveness was matrix dependent. Chemical treatment with medium chain fatty acids or a commercial formaldehyde product was most effective at mitigating *Salmonella* in rendered protein meals.

Keywords: *Salmonella,* chemical treatment, feed ingredients, feed safety

Background

Salmonella cross-contamination of ingredients is a major concern in the feed and rendering industries. In the United States alone, 11.2×10^9 lbs of protein and 10.9×10^9 lbs of fat are produced each year, of which 85% is used in animal feed ingredients [1]. The first documented case of *Salmonella* contamination in animal feed was as far back as 1948 [2]. Due to the historical occurrence of *Salmonella* in animal feed, the United States Food and Drug Administration carried out surveys of pathogen contamination in animal-based rendering plants across the United States. Of the 101 animal-based protein samples collected in 1993, 56% tested positive for *Salmonella* enterica [3]. As a follow-up, finished feed samples from feed mills and on-site farms were tested in 1994, and FDA reported that 25% of the 89 samples tested were positive for S. enterica [3]. Since then, other studies have shown similar results, including one

where 85% of 165 samples tested were positive for gram negative bacteria and 10% were positive for *Salmonella* [4]. While *Salmonella* may be perceived as a lower risk hazard in animal feed, *Salmonellosis* of animals has been linked to human illness [5]. If *Salmonella* contamination exists in animal feed or ingredients, it should be mitigated to minimize the risk to animal or human health.

Potential methods of bacterial contaminant mitigation can be characterized as thermal or non-thermal in nature. While thermal mitigation is an attractive option because it does not require the introduction of foreign compounds, it is a point in time strategy that does not eliminate the chance for recontamination [6]. For example, Binter et al (2011) demonstrated that up to 86% of thermally-processed samples collected from pellet coolers tested positive for *Salmonella* [7]. Alternatively, non-thermal mitigation methods may include the use of chemicals, such as organic acids (OA), formaldehyde, medium chain fatty acids (MCFA), essential oils (EO), and sodium bisulfate [6]. Of these, the most common feed additive is OA, particularly propionic, formic, lactic, and acetic acids. All of these OA have shown to be effective at reducing the concentration of *Salmonella* [8, 9, 10, 11]. Another chemical additive that is approved for the mitigation of *Salmonella* in animal feed is 0.03% formaldehyde [12]. Some EO, such as oregano and rosemary oils, have also been used to mitigate *Salmonella* to reduce the bacterial load by 1-2 log10 CFU/g in food products [13,14]. Medium chain fatty acids, such as caprylic and capric acid, have also shown to be potential *Salmonella* mitigants by damaging the cell membrane of the bacteria [15]. While there is some data available on the mitigation potential of particular chemicals against *Salmonella* inoculum, very little research has evaluated the ability of chemical treatment of various feed ingredients to prevent cross-contamination with the bacteria. Because various physical states, nutrient composition, and properties of each chemical

additive and feed matrix are different, each chemical may interact differently as a mitigants. Therefore, the objective of this experiment was to evaluate the effectiveness of various chemical treatments to mitigate post-processing *Salmonella* contamination in feed ingredients.

Results

All main effects and interactions were highly significant ($P < 0.001$; Table 3.4). Overall, the MCFA, commercial formaldehyde product, OA, and EO treatments each had a lower concentration of *Salmonella* compared to the control ($P < 0.05$). The MCFA treatment and commercial formaldehyde product were the most successful at preventing cross-contamination from *Salmonella* (0.51 and 0.65 CFU/g, respectively; Table 3.5), which were more successful than the OA treatment (1.20 CFU/g) or EO treatment (2.10 CFU/g). The sodium bisulfate treatment was similar to the control ($P = 0.14$; 2.38 vs. 2.56 CFU/g).

Differences were also observed when evaluating the main effect of feed matrix (Table 3.6). Values between the avian blood meal and porcine meat and bone meal were similar ($P = 0.36$; 1.73 vs. 1.82 CFU/g, respectively), but were less successful at preventing cross-contamination of *Salmonella* than feather meal or poultry by-product meal ($P < 0.05$; 1.36 vs. 1.36 CFU/g).

Time also played a major role in the degradation of *Salmonella*. Over the 42 days of the experiment, the quantity amount of *Salmonella* detected decreased linearly ($P < 0.05$; 4.50, 2.65, 1.75, 0.95, 0.49, 0.50, and 0.13 CFU/g for 0, 1, 3, 7, 14, 21, and 42, respectively; Table 3.7). With the exception of d 14 and 21 ($P = 0.93$), the quantity of *Salmonella* detected each day differed from one another ($P < 0.05$).

The MCFA mixture was the most effective chemical treatment in the avian blood meal and feather meal, followed by the commercial formaldehyde treatment. The commercial

formaldehyde treatment was the most successful mitigant in the poultry by-product meal and meat and bone meal, followed by the MCFA and OA treatments ((P < 0.05; Table 3.8).

When evaluating efficacy over time, the MCFA and commercial formaldehyde treatments were the most effective at mitigating *Salmonella* during the entire experimental period ((P < 0.05; Table 3.9), particularly over the days soon after treatment and inoculation. The OA treatment was also effective at mitigating *Salmonella* over the experimental period, but required more time for effectiveness than the MCFA or commercial formaldehyde treatments((P < 0.05). Interestingly, the EO and SBS treatments were similar to the untreated control during the duration of the 42-d experiment.

Feed matrix had a significant impact on the *Salmonella* concentration over the 42 day analysis period. The *Salmonella* concentration of feather meal was lower (P < 0.05) than the other feed matrices on d 0 and 1post inoculation. However, poultry by-product meal had a lower (P < 0.05) *Salmonella* concentration than the other matrices from 3 to 42d after inoculation (Table 3.10). Interestingly, we observed the blood meal and meat and bone meal still had residual levels of *Salmonella* by the end of the 42-d experimental period, while the blood meal and feather meal matrices self-mitigated over time.

Discussion

The purpose of this proof-of-concept experiment was to evaluate if categories of chemical treatments could prevent post-processing *Salmonella* contamination, which was determined by quantifying the concentration of *Salmonella* colonies present by XLD plating. Surprisingly, the MCFA mixture performed similar to the commercial formaldehyde product. The commercial formaldehyde product used in this experiment is intended to inhibit mold growth, and has been shown to maintain feed and feed ingredients in *Salmonella* negative form

[17]. The product is used in the animal feed industry to prevent recontamination in the manufacturing, storage, and transportation of animal feed or feed ingredients [17]. Meanwhile, MCFA, such as capric and caprylic acid have been shown to be effective against *E. coli* and *Salmonella* growth [15]. Caprylic acid added to feed has been shown to decrease the quantity of *Salmonella* colonization in broiler chicks [18]. While the added concentration of MCFA in that experiment was 0.7 and 1%, it was nearly double that concentration in our experiment because we were testing a proof-of-concept to first assess if an extremely high combination of chemicals in a single chemical category was effective at preventing *Salmonella* cross-contamination. We wholly recognize that our tested levels are not realistic inclusion levels for animal feed, but these results provide a direction for future research emphasis. According to our findings, more research is warranted to identify the mode of action of MCFA at preventing cross-contamination of *Salmonella* in animal feed, as well as elucidate the effectiveness of both lower doses and single MCFA inclusion levels.

This research confirmed that MCFA were more antibacterial that OA, a concept that has been previously reported [19]. While less effective than MCFA or formaldehyde treatment, the inclusion of the OA blend in rendered ingredients was still effective at preventing *Salmonella* post-processing contamination compared to the control. Previous research supports the bactericidal activity of OA. Propionic acid has been shown to destroy 90% of the cell population within 1 h and formic acid within 3 h of treatment [20]. The combination of propionic and formic acids in a blend evaluated by Carrique-Mas et al. (1991) performed similarly as the OA treatment in this study [20], and was previously reported to be less successful than a formaldehyde control [21]. The proposed mode of action of OA treatment to mitigate *Salmonella* contamination suggests that OA penetrate the cell membrane and enter the bacterial cell's cytoplasm, where

they dissociate causing the pH of the cell to increase, causing cell atrophy [22]. There are further advantages to OA treatment compared to formaldehyde because OA is proposed to be relatively stable in feed and can occur naturally in living organisms, and therefore may have greater consumer appeal when listed on an ingredient label [23].

Another consumer-friendly chemical additive that was effective at decreasing the risk of *Salmonella* cross-contamination compared to the control are EO. Previous research supports our findings that EO is effective at mitigating *Salmonella*. Garlic and oregano have both been shown to be effective at mitigating *Salmonella*, and have minimum inhibitory concentrations of 729 and 417 ppm and maximal tolerated concentrations of 52 and 104 ppm, respectively [24]. Rosemary has also been shown to be effective against *Salmonella* contamination with a minimum inhibitory concentration of 0.3 % v/v and minimal bactericidal concentrations of 0.5% v/v against E.coli contamination [25]. The phenolic compounds in EO are proposed as essential to their mode of action as bactericidal compounds [26]. Some EO can contain phenol compounds that are thought to interact with and disrupt the cell membrane of bacteria, causing the cell to lose functional properties and leak the inner cell materials [26]. The EO treatment in this study was effective, but not to the same magnitude as MCFA, formaldehyde, or OA inclusion. Still, its effectiveness was demonstrated compared to the control, and may vary within different targeted ingredients.

The sodium bisulfate treatment was evaluated due to its commercial availability in the pet food and poultry industries. The chemical additive has acidulate, and desiccant properties, but is in a granular form that makes it attractive to utilize within dry bulk manufacturing systems, such as animal feed mills [27]. However, the addition of the product did not prevent *Salmonella* post-processing contamination of the tested ingredients compared to the control Potentially, this dry

powder form was partially responsible for the product's lack of mitigant properties in this experiment because the granular form is more challenging to effectively coat ingredient particles, which reduces the likelihood of the product contacting *Salmonella* cells. If a smaller particle size or liquid addition of the product would have been used, it may have been more successful mitigation. This concept could apply to all solid-phase mitigants, suggesting that liquid- or gaseous-phase chemical additives may be more effective at preventing *Salmonella* mitigation due to their improved coating characteristics.

Conclusions

Time, MCFA, commercial formaldehyde product, OA, and EO all decreased the presence of *Salmonella* in feed ingredients, but those results can vary based on the feed ingredient. *Salmonella* was relatively stable in the avian blood meal over 42 days, compared to 21 days in the other three feed ingredients. The MCFA and formaldehyde treatments were most effective at preventing post-processing contamination of rendered protein meals. Further research is needed to evaluate the effectiveness of MCFA inclusion at more practical inclusion levels.

Methods

Chemical Treatment

Six chemical treatments were applied to four different feed matrices. The chemical treatments included: 1) *Salmonella* positive with no chemical addition, 2) 0.3% wt/wt commercial formaldehyde product (Termin-8, Anitox Corp, Lawrenceville, GA), 3) 2% wt/wt EO blend [1:1 ratio of garlic oleoresin, turmeric oleoresin, capsicum oleoresin, rosemary extract, and wild oregano essential oils], 4) 3% wt/wt OA blend [1:1 ratio of lactic, propionic, formic, and benzoic], 5) 2% wt/wt MCFA blend [1:1 ratio of caproic, caprylic, and capric acids], and 6)

1% sodium bisulfate (Jones-Hamilton Co, Walbridge, OH).). The 4 matrices included: 1) feather meal, 2) avian blood meal, 3) porcine meat and bone meal, and 4) poultry by-product meal. Matrices had not be previously treated with other chemicals, and were analyzed for proximate analysis, fatty acid composition, and amino acid composition (Tables 3.1,3.2, and 3.3). One kilogram (kg) of each feed matrix was placed in a lab scale ribbon mixer where the liquid chemicals were fogged into the feed and the dry powder treatment was mixed directly into the mixer.

Inoculation Preparation

A total of 100 µL of *Salmonella* enterica subsp. enterica Serovar Typhimurium (ATCC14028) was placed into 10 mL of trypticase soy broth (TSB; Difco, Beston, Dickinson and Company, Franklin Lakes, NJ) and grown for 24 h at 35°C. The culture was then centrifuged at 5000×g. Next, 7 mL of the TSB supernatant was removed. The remaining 3mL of supernatant was then vortexed to remove cells from the side of the tube and then used for the inoculation.

Feed Ingredient Inoculation

A total of 120 g of each chemically-treated matrix was weighed and placed in plastic a total of 24 containers for inoculation. A pump spray nozzle was then used to disperse the cells across each matrix. The pump nozzle was first cleaned using ethanol, and then TSB was used to flush out the pump. Following the cleaning step, the spray nozzle was placed into the 3ml of *Salmonella* cells which were then applied to the feed treatments. Once the inoculum was added, each container was shaken to mix in the inoculum throughout the matrix. Each inoculated matrix was then stored in containers at room temperature throughout the 42 day experiment. On each analysis day, the containers were opened inside a hood to prevent outside contamination.

Microbiological Analysis

On each analysis day three samples were taken from each container. A total of 11 g per sample were placed into 99 ml of BPW and mixed. Samples were then diluted to 103, 102, and 101 and plated on Xylose lysine deoxycholate agar (XLD, Difco, Beston, Dickinson and Company, Franklin Lakes, NJ), with a limit of detection of less than 100 CFU/g of feed matrix. Procedures were repeated on d 0, 1, 3, 7, 14, 21 and 42 to evaluate chemical effectiveness over time.

Statistical Analysis

Data were analyzed using the GLIMMIX procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC) after log transformation with the fixed effects of chemical treatment and feed matrix with day as a repeated measure. There were 3 replicates of each chemical treatment × feed matrix combination at each sampling day. Differences were considered statistically significant at $P < 0.05$.

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Figures and Tables

Table 3.1 Proximate analysis of feed matrix (as-is basis)

Item[1,2]	Feather meal	Blood meal	Meat and bone meal	Poultry by-product meal
Moisture	3.42	9.14	3.06	5.17
Crude fat	6.45	0.53	11.10	12.84
Crude fiber	0.78	0.45	1.66	1.42
Crude Protein	88.53	87.17	56.42	62.25
Ash	1.40	2.20	26.76	13.15
Calcium	0.31	0.12	9.34	3.44
Phosphorus	0.20	0.32	4.72	2.15

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

Table 3.2 Fatty acid analysis of feed matrix (as-is basis)

Item[1,2]	Feather meal	Blood meal	Meat and bone meal	Poultry by-product meal
Myristic (14:0)	1.44	2.84	1.48	1.10
Myristoleic (9c-14:1)	0.17	0.20	0.25	0.39
C15:0	0.17	0.18	0.10	0.16
Palmitic (16:0)	25.04	23.57	26.01	23.23
Palmitoleic (9c-16:1)	5.02	2.23	3.35	5.31
Margaric (17:0)	0.85	0.48	0.36	0.25
10c-17:1	0.17	0.08	0.47	0.20
Stearic (18:0)	8.80	16.24	14.14	8.09
Elaidic (9t-18:1)	1.39	11.04	0.56	0.82
Oleic (9c-18:1)	34.14	19.51	40.31	33.35
Vaccenic (11c-18:1)	0.00	0.00	0.00	0.00
Linoleic (18:2n6)	13.28	7.49	5.02	17.52
Linolenic (18:3n3)	0.56	1.27	0.14	0.74
Stearidonic (18:4n3)	0.00	0.00	0.00	0.00
Arachidic (20:0)	1.47	0.72	0.25	0.30
Gonodic (20:1n9)	0.59	0.48	1.06	0.51
Homo-a-linolenic(20:3n3)	0.00	0.00	0.00	0.00
Arachidonic [20:4n6]	0.53	0.75	0.41	0.94
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)	0.63	0.77	0.19	0.29
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)	0.08	0.16	0.24	0.34
Lignoceric (24:0)	0.46	0.84	0.70	1.59
Nervonic (24:1n9)	0.00	0.68	0.13	0.00

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

Table 3.3 Amino acid profile of feed matrix (as-is basis)

Item[1,2]	Feather meal	Blood meal	Meat and bone meal	Poultry by-product meal
Taurine	0.02	0.01	0.10	0.43
Hydroxyproline	0.03	0.00	2.94	2.34
Aspartic Acid	5.77	7.52	4.18	5.01
Threonine	4.22	4.14	1.83	2.35
Serine	8.97	3.59	2.02	2.24
Glutamic Acid	8.67	8.02	6.63	7.49
Proline	9.02	3.84	4.67	4.39
Lanthionine	1.43	0.19	0.19	0.00
Glycine	6.69	3.40	7.40	6.67
Alanine	3.88	5.96	3.98	4.17
Cysteine	5.18	1.64	0.49	0.69
Valine	6.74	5.14	2.32	2.83
Methionine	0.56	1.04	0.79	1.18
Isoleucine	4.37	3.21	1.65	2.31
Leucine	7.32	8.76	3.42	4.28
Tyrosine	2.64	2.78	1.46	2.04
Phenylalanine	4.47	5.18	1.93	2.58
Hydroxylysine	0.02	0.05	0.35	0.36
Ornithine	0.62	0.12	0.13	0.17
Lysine	1.81	6.68	3.06	3.92
Histidine	0.79	4.36	1.17	1.34
Arginine	6.26	4.54	4.02	4.35
Tryptophan	0.27	0.57	0.35	0.50
Total	89.75	80.74	55.08	61.64

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

Table 3.4 Main effects and interaction on *Salmonella* quantity.

Effect	*P* =
Treatment	< 0.001
Feed matrix	< 0.001
Day	< 0.001
Treatment × Feed matrix	< 0.001
Treatment × Day	< 0.001
Feed matrix × Day	< 0.001
Treatment × Feed matrix × Day	< 0.001

Table 3.5 Treatment main effects for chemical means for chemically treated *Salmonella* inoculated feed matrices

	Chemical Treatment							
Item[1]	Untreated positive control	Commercial formaldehyde	Essential oil	Medium chains	Organic acid	Sodium bisulfate	SEM	*P*=
CFU/g[2]	2.56 [a]	0.65 [d]	2.10 [b]	0.51 [d]	1.20[c]	2.38 [a]	0.08442	< 0.0001

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[2]Values are represented by $\log_{10}$ colony forming units per gram.

Table 3.6 Treatment main effects for feed matrix means for chemically treated *Salmonella* inoculated feed matrices

	Feed Matrix					
Item[1]	Avian blood Meal	Feather meal	Porcine meat and bone meal	Poultry by-product meal	SEM	*P*=
CFU/g[2]	1.73 [a]	1.36 [b]	1.82[a]	1.36 [b]	0.06893	< 0.0001

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[2]Values are represented by $\log_{10}$ colony forming units per gram

Table 3.7 Treatment main effects for day means for chemically treated *Salmonella* inoculated feed matrices

	Day								
Item[1]	0	1	3	7	14	21	42	SEM	*P* =
CFU/g[2]	4.50 [a]	2.65 [b]	1.75 [c]	0.95 [d]	0.49 [e]	0.50 [e]	0.13 [f]	0.09118	< 0.0001

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[2]Values are represented by $\log_{10}$ colony forming units per gram

Table 3.8 Treatment × feed matrix interaction for chemically treated *Salmonella* inoculated feed matrices

Item[1,2]	*Salmonella* +	Commercial formaldehyde	Essential oil	Medium chain fatty acid	Organic acid	Sodium bisulfate	SEM	*P*=
Blood meal	3.28 [a]	0.72 [ij]	1.36 [gh]	0.54 [ijk]	1.54 [fgh]	2.91 [ab]	0.1688	< 0.0001
Feather meal	2.68 [bc]	0.32 [j]	2.09 [de]	0.21 [k]	0.47 [ijk]	2.40 [cd]		
Meat/bone meal	2.38 [cd]	0.82 [i]	3.19 [a]	0.54 [ijk]	1.49 [fgh]	2.46 [bcd]		
Poultry by-product	1.90 [ef]	0.73 [ij]	1.75 [efgh]	0.73 [ij]	1.30 [h]	1.77 [efg]		

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[2]Values are represented by $\log_{10}$ colony forming units per gram

Table 3.9 Treatment × day interaction for chemically treated *Salmonella* inoculated feed matrices

	Day								
Item[1,2,3]	0	1	3	7	14	21	42	SEM	*P* =
Salmonella +	5.45 [a]	4.55 [c]	3.12 [ef]	2.42 [gh]	1.02 [jk]	1.19 [jk]	0.19 [lm]	0.2234	< 0.0001
Commercial form.	3.57 [e]	0.33 [lm]	UND [m]	UND [m]	0.26 [lm]	0.37 [lm]	UND [m]		
Essential oils	5.22 [ab]	3.71 [de]	2.88 [fg]	1.45 [ij]	0.71 [kl]	0.36 [lm]	0.36 [lm]		
Organic acids	4.64 [bc]	2.44 [gh]	1.14 [jk]	UND [m]	0.17 [lm]	UND [m]	UND [m]		
Medium chains	2.35 [gh]	0.66 [kl]	0.17 [lm]	UND [m]	UND [m]	0.36 [lm]	UND [m]		
Sodium bisulfate	5.75 [a]	4.21 [cd]	3.16 [ef]	1.85 [hi]	0.77 [kl]	0.72 [kl]	0.23 [lm]		

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1]UND, undetectable (counts that averaged less than 100 CFU/g had its log reported as such).

[2] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[3]Values are represented by $\log_{10}$ colony forming units per gram

Table 3.10 Feed matrix × day interaction for chemically treated *Salmonella* inoculated feed matrices

	Day								
Item[1,2,3]	0	1	3	7	14	21	42	SEM	*P* =
Blood meal	4.85 [a]	2.85 [c]	1.87 [ef]	1.27 [gh]	0.52 [jkl]	0.60 [ijkl]	0.13 [lm]	0.1824	< 0.0001
Feather meal	3.41 [b]	2.12 [e]	1.58 [fg]	1.06 [hi]	0.48 [klm]	0.86 [hijk]	UND [m]		
Meat/bone meal	4.86 [a]	2.77 [cd]	2.31 [de]	1.00 [hij]	0.84 [hijk]	0.53 [jkl]	0.39 [klm]		
Poultry by-product	4.87 [a]	2.86 [c]	1.22 [gh]	0.48 [klm]	0.11 [lm]	UND [m]	UND [m]		

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1]UND, undetectable (counts that averaged less than 100 CFU/g had its log reported as such).

[2] Four feed matrices were treated with six different chemical treatments and inoculated with *Salmonella* enterica subsp. enterica Serovar Typhimurium and plated on XLD over 42 days.

[3]Values are represented by $\log_{10}$ colony forming units per gram

Chapter 4 - Mitigation of a *Salmonella* Surrogate in Poultry Feed using a Dry Acidulant

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Summary

Heat treatment is used in the feed industry to mitigate pathogens, but it serves as a point in time strategy that does not eliminate the chance for recontamination in the manufacturing process. An experiment was conducted to evaluate the effectiveness of a dry granular acid, sodium bisulfate (SBS; Jones-Hamilton, Co., Waldridge, OH), to mitigate contamination of *Salmonella* in poultry feed. A surrogate organism, *Enterococcus faecium* (*E. faecium*), was utilized for this research. Treatments were arranged in a 2 × 6 factorial with two diet forms (non-processed mash vs pelleted feed) and 6 levels of SBS (0, 0.175, 0.35, 0.70, 1.4, and 2.8% w/w). A standard, broiler grower diet was inoculated with *E. faecium*, treatments mixed with SBS, and then either retained as mash or pelleted. Mash samples prior to thermal processing and corresponding pelleted samples were collected. Samples were analyzed for *E. faecium* on d 0, 2, 4, 7, and 14. Both diet form and SBS inclusion level, as well as their interaction, affected pathogen concentrations. Specifically, pelleting resulted in a 3-log reduction in *E. faecium* (5.82 vs. 2.36 CFU/g for mash vs. pelleted feeds, respectively). In both pelleted and mash diets, there was a linear decline in *E. faecium* with increasing SBS inclusion in mash diets. There was also a

linear decrease in *E. faecium* over time. In summary, this research suggests that thermal processing, SBS concentration, and time all impact biological pathogen levels in poultry diets, and that including a dry granular acid may be an effective method to reduce pathogen risk.

Description of Problem

The U.S. feed industry continues to set the standard for safe and nutritionally-adequate animal feed production. Still, recent reports of *Salmonella* in animal feed and ingredients demonstrate the necessity for feed mills and animal feeders to consider their role in the reduction of biological pathogens in the farm to fork system [1]. Surveillance of animal and vegetable protein ingredients conducted by the Food and Drug Administrations (FDA) report that rate of *Salmonella* contamination has decreased from 49.7 to 22.9% contamination rate between 1993 and 2013 [2, 3]. This is a substantial reduction, and ingredient suppliers and the feed industry should be commended for their proactive adoption of mitigation methods to reduce *Salmonella* occurrence. However, the current contamination rate of more than 1 in 5 samples demonstrates there is still opportunity for improvement of feed safety practices. Thermal processing by pelleting is commonly used to mitigate pathogens within animal feed [1], and research supports that pellet mill conditioning temperatures of at least 80 to 85°C help mitigate bacterial contamination [4]. However, thermal processing is a point-in-time mitigation strategy that does not prevent recontamination within the subsequent cooling, conveying, load-out, and transportation process.

The inclusion of chemical additives in feed, such as organic acids or formaldehyde, has demonstrated successful mitigation properties for biological pathogens [5]. Specifically, organic acids mitigate pathogens by dissociating and passing through the lipid membrane of the bacterial cell, causing the disruption in the cellular pH gradients and intracellular regulation [6].

Meanwhile, mitigation via formaldehyde results in irreversibly cross-linking of proteins and is commonly used to improve hygiene within the poultry and feed industry [7]. However, chemical additives have drawbacks because of potential worker health concerns and specialized equipment required for successful application. Some chemicals, including formaldehyde, can also volatize over time if stored improperly [8]. Liquid chemical additives are particularly challenging because feed is generally manufactured in dry bulk form, and many feed mills do not have the capability to adequately incorporate liquids. Thus, the inclusion of a dry acidulant may lend successful mitigation properties observed in other chemical additives, but in a more convenient dry form for easier application. Sodium bisulfate is currently used as an acidifier to increase enzyme activity in the poultry industry; however, the acidic nature of SBS could potential alter the pH of the feed causing the bacteria to become susceptible to its desiccant properties.
Thus the objectives of this experiment were to: 1) determine the minimum effective concentration of sodium bisulfate needed to reduce the *Salmonella*-surrogate *E. faecium* in poultry mash and pelleted poultry feed by one log, 2) determine the effectiveness of various levels of sodium bisulfate necessary to maintain feed *Salmonella*-surrogate *E. faecium* free over time, and 3) determine the effect of pelleting vs mash and the interactive effects of SBS and feed processing on the *Salmonella*-surrogate *E. faecium*.

Materials and Methods

Basal Diet Mixing

A single basal broiler chick grower diet was manufactured in the biosafety level-2 Cargill Feed Safety Research Center in the O.H. Kruse Feed Technology Innovation Center at Kansas State University (Table 4.1). The diet was divided into 22.7-kg batches for each of 6 treatments

and 3 replicates/treatment for a total of 18 batches. The 6 treatments included feed which was: 1) *E. faecium*-inoculated and contained 0% SBS, 2) *E. faecium*-inoculated with 0.175% SBS, 3) *E. faecium*-inoculated with 0.35% SBS, 4) *E. faecium*-inoculated with 0.70% SBS, 5) *E. faecium*-inoculated with 1.40% SBS, and 6) *E. faecium*-inoculated with 2.80% SBS. There were three reps per treatment, and treatments were randomized within rep. Mash and hot pellet samples were collected and stored on ice in waterproof and airtight containers to prevent cross-contamination during processing.

Inoculum Preparation and Feed Inoculation

Enterococcus faecium (ATCC 8459) has been shown to serve as a suitable *Salmonella*-surrogate in thermal inactivation studies in the food industry and most recently in an extrusion setting [9, 10, 11]. In our experiment, the pathogen was transferred from a trypticase soy agar plate (TSA, Difco, Beston, Dickinson and Company, Franklin Lakes, NJ) and placed into 10 mL of trypticase soy broth (TSB, Difco, Beston, Dickinson and Company, Franklin Lakes, NJ) and grown for 48 h at 35°C. After the initial culture activation, the culture was then propagated through ensuing transfers to new TSB to produce a total of 4 L of growing *E. faecium*.
The basal diet was mixed with 4 L of *E. faceium* inoculum by spray application for a total wet mix time of 10 minutes using a Davis paddle mixer (model S-3, H. C. Davis & Sons Manufacturing Inc., Bonner Springs, KS).

Sodium Bisulfate Addition and Pelleting

After inoculation with *E. faecium*, treatment diet manufacturing order was randomized within rep and mixed using a 45 kg mixer for 3 minutes. Non-thermally processed mash samples were collected after mixing with SBS and stored on ice in waterproof, air-tight containers. Diets

were pelleted using a pellet mill (CL Type 5, California Pellet Mill, Crawfordsville, IN) with conditioning temperatures ranging from 70 to 73°C hot mash temperature. An un-inoculated diet with 0% SBS was utilized as a flush between each treatment within each pelleting treatment. Hot pellet samples from each diet were collected, cooled by a pilot scale cooler to room temperature, and placed in waterproof, air-tight containers. All samples were then transported to the Kansas State University Microbiology and Toxicology Laboratory for analysis.

Microbiological Analysis

E. faecium in the mash and pelleted feed was enumerated via serial dilution in buffered peptone water (BPW; Beston, Dickinson and Company, Franklin Lakes, NJ) and plated on the selective and differential m-*Enterococcus* agar (m-ENT; Becton, Dickinson, and Company, Franklin Lakes, NJ). All inoculated plates were incubated at 35°C for 48 h. After incubation, pink-purple colonies, typical of *Enterococcus,* were counted and colony forming units (CFU) per g were determined. Procedures were repeated on d 0, 2, 4, 7, and 14 after processing to evaluate SBS effectiveness over time.

Statistical Analysis

Results were analyzed using the GLIMMIX procedure of SAS v. 9.3 (SAS Inst. Inc., Cary, NC) after log transformation with the fixed effects of form (mash or pellet), SBS inclusion level (0, 0.175, 0.35, 0.70, 1.40, or 2.80%), and time (0, 2, 4, 7, or 14 d), with time serving as a repeated measure. All interactions were evaluated, but only the form × day interaction was significant, so all other interactions ($P > 0.87$) were removed from the model. Linear and quadratic models were also utilized for the statistical analysis.

Results and Discussion

As expected, pelleting resulted in an overall reduction in *E. faecium* compared to mash diets, but the 3-log magnitude of reduction was surprising (Table 4.2; $P < 0.0001$). Previously, Himathongkham et al. (1996) observed only a 2-log reduction of *Salmonella enteriditis* in poultry feed under similar pelleting conditions [12]. The inconsistency may have been due to differences in diet formulation, processing, or pathogen resulting from our use of a surrogate instead of the same *Salmonella* strain. Regardless, these data demonstrate that, while pelleting reduced the concentration of the pathogen, bacterial contamination was still present. Potentially, either pelleting did not completely destroy all bacteria or cross-contamination during the cooling process re-contaminated the pelleted samples. The inoculated pelleted samples were intentionally cooled in a common cooler to mimic traditional cooling in a feed mill because the manner in which pelleted feed was contaminated was less important than the fact that they still tested positive for bacterial contamination and could therefore be a potential vector for transmission.

Others have observed similar contamination rates after pelleting feeds naturally afflicted with bacterial contaminants. Various researchers have demonstrated that pelleting reduced contamination rates of naturally contaminated feeds and ingredients by 50 to 93% [4, 13, 14]. Conditioning temperature and time, initial contamination concentration, and moisture all affect the successful mitigation of bacterial pathogens in animal feed [4]. Thus, these can all be altered to further reduce the risk of biological contamination.

While the overall main effect of SBS inclusion was not significant (Table 4.3; $P = 0.17$), increasing SBS inclusion reduced *E. faecium* concentrations in a linear manner ($P = 0.02$). Sodium bisulfate is a dry acid used as an acidifier in the poultry feed industry to increase enzyme activity and therefore feed efficiency of diets. SBS is recognized as a general purpose feed

additive by the Association of American Feed Control Officials [15, 16, 17]. This dry acid is suspected to be a potential mitigant of bacterial pathogens because of its two-fold properties as an acidulant and desiccant and is desirable because of its dry form. The acidic nature of SBS alters the pH of the feed which reduces the likelihood of bacterial re-contamination or proliferation. Furthermore, the acidulant action of SBS may cause bacteria to become more susceptible to its desiccant properties, which then destroy existing bacterial cells in the feed by extracting water from the bacteria that impairs the intracellular function and compromising survivability. Previous research in our laboratory has evaluated SBS powder as a coating for cat and dog food, where we reported its inclusion at 0.2 to 0.8% resulted in a 1.0- and 1.5-log reduction in *Salmonella* for cat and dog food, respectively, over a 14 d experiment [18]. While the main effect of SBS granular inclusion in the current experiment was not significant, the linear improvement with increasing concentrations suggest further research with the powder form is warranted to evaluate its use as a chemical mitigant for bacterial pathogens in the animal feed industry. The poultry industry currently uses the granular form of SBS in feed for feed efficiency improvement. Therefore, the granular form was used in this study instead of the powder form. The powder form would significantly increase the surface area and distribution throughout the feed.

As expected, *E. faecium* concentrations declined over time in a linear manner (Table 4.4; $P = 0.001$). These differences were predominantly driven by reduced *E. faecium* concentrations on d 7 and 14 compared to d 0 ($P < 0.05$), which were similar to those reported by Jeffery et al. (2015) [18]. Based on these findings, it is therefore not surprising that there was an interaction ($P = 0.04$) between feed form and time, where pelleted diets had reduced *E. faecium* concentrations by more than 3-logs compared to mash diets on d 0. In contrast, concentrations in pelleted diets

remained relatively consistent over time while the concentration of *E. faecium* in mash diets decreased (Figure 4.1). This could be due to the pellet not receiving the same processing temperature throughout the pellet.

In summary, this research suggests that thermal processing and time were the most successful mitigation methods, but that increasing sodium bisulfate inclusion also reduces pathogenic bacteria contamination in poultry feed in a linear manner.

Conclusions and Applications

1. Pelleting at 70°C resulted in a 3-log reduction of *E. faecium* concentrations in a broiler grower diet. However, pathogen contamination still existed after thermal processing, demonstrating that the potential still exists for pelleted feed to serve as a vector of bacterial pathogen transmission.

2. The lowest tested inclusion level of SBS granules, 0.175%, resulted in a 1-log reduction of *E. faecium* in the mash samples. However, the difference was not significantly different from the control or other tested levels. Still, SBS inclusion reduced pathogen concentrations in a linear manner.

3. The concentration of *E. faecium* decreased over time, but SBS granule inclusion did not further mitigate the pathogen contamination.

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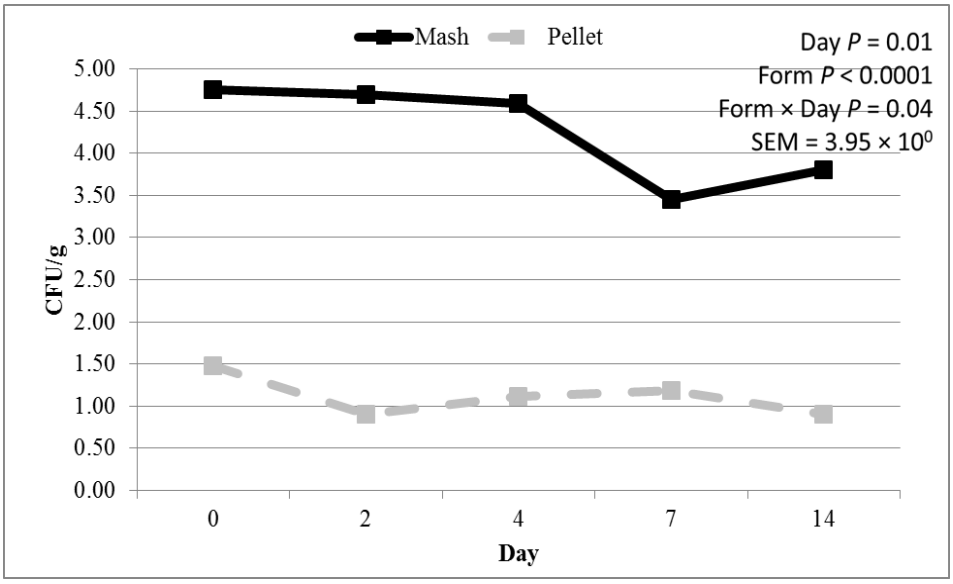
Figures and Tables

Figure 4.1 Effect of form (mash vs pellet) and time on *E. faecium* concentration



[1]A standard mash broiler grower diet was treated with varying levels of a commercially-available feed acidulant, sodium bisulfate, and then inoculated with *Enterococcus faecium*, a surrogate for *Salmonella*. Treatments were maintained in mash form or pelleted at 70°C and analyzed for *E. faecium* on d 0, 2, 4, 7, and 14. Pelleting reduced *E. faecium* concentrations by approximately 3.5-logs initially, but then *E. faecium* levels remained relatively constant in pelleted feeds between d 0 and 14. Conversely, the *E. faecium* concentrations in mash diets were reduced over the experimental period, particularly from d 4 to 7.

Table 4.1 Formulated nutrient composition of the poultry grower diet

Ingredient, %	
Ground corn	62.65
Soybean meal, 48%	21.10
Poultry byproduct meal	9.00
Soybean oil	3.60
Limestone	1.38
Dicalcium phosphate, 21% P	1.30
Salt	0.26
L-Lys-HCl	0.24
DL-Methionine	0.22
Vitamin and mineral premix[1]	0.25
Total	100.0
Calculated analyses	
ME, kcal/kg	3,016
CP, %	20.00
Ca, %	0.85
Available P, %	0.40
Total Lys, %	1.10
Total Met + Cys, %	0.83

[1]The premix supplied the following per kilogram of feed: vitamin A, 6,601; cholecalciferol, 1,980 IU; niacin, 55 mg; α-tocopherol, 33 mg; pantothenic acid, 11 mg; riboflavin, 6.6 mg; pyridoxine, 4 mg; menadione, 2 mg; thiamine, 2 mg; folic acid, 1.1 mg; biotin, 0.13 mg; vitamin B_{12}, 0.02 mg; Zn, 120 mg; Mn, 120 mg; Fe, 80 mg; Cu, 10 mb; I, 2.5 mg; Co, 1.0 mg, and Se, 0.2 ppm.

Table 4.2 The effect of feed form on concentrations of *E. faecium* in poultry feed

Item[1]	*E. faecium*, CFU/g
Mash	4.26[a]
Pellet	1.12[b]
SEM	0.115
	P-value
Form	< 0.0001

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1]A standard mash broiler grower diet was treated with varying levels of a commercially-available feed acidulant, sodium bisulfate, and then inoculated with *Enterococcus faecium*, a surrogate for *Salmonella*. Treatments were maintained in mash form or pelleted at 70°C and analyzed for *E.faecium* on d 0, 2, 4, 7, and 14.

Table 4.3 The effect of sodium bisulfate inclusion on concentrations of *E. faecium* in poultry feed

Item[1]	*E. faecium*, CFU/g
Sodium bisulfate level	
0.00%	2.78[ab]
0.175%	3.03[a]
0.35%	2.79[ab]
0.70%	2.64[ab]
1.40%	2.62[ab]
2.80%	2.27[b]
SEM	0.199
	P-value
Level	0.17
Linear	0.02
Quadratic	0.27

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1]A standard mash broiler grower diet was treated with varying levels of a commercially-available feed acidulant, sodium bisulfate, and then inoculated with *Enterococcus faecium*, a surrogate for *Salmonella*. Treatments were maintained in mash form or pelleted at 70°C and analyzed for *E.faecium* on d 0, 2, 4, 7, and 14.

Table 4.4 The effect of time on concentrations of *E. faecium* in poultry feed

Item[1]	*E. faecium*, CFU/g
Day	
0	3.11[a]
2	2.80[abc]
4	2.85[ab]
7	2.32[c]
14	2.36[bc]
SEM	0.182
	P-value
Day	0.01
Linear	0.0008
Quadratic	0.87

[ab]Values in columns not sharing the same superscript letter are significantly different ($P \leq 0.05$).

[1]A standard mash broiler grower diet was treated with varying levels of a commercially-available feed acidulant, sodium bisulfate, and then inoculated with *Enterococcus faecium*, a surrogate for *Salmonella*. Treatments were maintained in mash form or pelleted at 70°C and analyzed for *E.faecium* on d 0, 2, 4, 7, and 14.