THE ROLE OF *SALMONELLA* IN ANIMAL FOOD

by

ANDREA JEFFREY

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Approved by:

Major Professor
Dr. Cassandra Jones

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Abstract

Salmonella contamination in animal food production facilities is a growing concern. The bacteria has been the cause of 40% of pet food recalls in the past 5 years, and there are potential human health implications because pet food is a direct human contact food. A potential method to reduce *Salmonella* contamination in pet food is through the use of acidifiers and desiccants to destroy and inhibit growth of bacteria. The objective of this thesis was to quantify *Salmonella* contamination in livestock feed and pet food manufacturing facilities, and propose mitigation measures to mitigate the presence of pathogens in animal food. Therefore, the objective of Experiment 1 was to investigate sources of *Salmonella* contamination throughout livestock feed (n = 2) and pet food (n = 2) manufacturing facilities on a specific sampling day. *Salmonella* was present in all four facilities. However, one of the livestock feed manufacturing facilities had more than double the *Salmonella*-positive locations than all other facilities. This experiment demonstrated that surface type and location should be taken into consideration when controlling *Salmonella* contamination. In Experiments 2 and 3, the use of a commercial powdered dry acidulant, sodium bisulfate, was studied as a coating of dog kibble to reduce and prevent *Salmonella* growth over time. The coating reduced *Salmonella* concentration, and its efficacy was not impacted by altering the bulk density or surface area of the kibble. Experiment 4 was conducted to determine the efficacy of sodium bisulfate added to poultry mash to reduce or prevent *Salmonella* growth over time. The inclusion of the dry acidulant did not reduce *Salmonella* concentration; however, storage time reduced *Salmonella* contamination in poultry feed. In summary, *Salmonella* contamination exists in manufacturing facilities, but the location and magnitude of contamination differs. Furthermore, sodium bisulfate effectively reduces *Salmonella* contamination when applied as a pet food coating, but not in poultry feed.

Table of Contents

List of Figures vii
List of Tables viii
Acknowledgements ix
Chapter 1 - Interaction between Surface Area and Pathogen Contamination in Animal Food 1
Introduction 1
Methods of Detecting Pathogens in Animal Food 3
Culture onto Selective Media 3
Immunomagnetic Separation 4
Enzyme Immunoassay 4
Enzyme-Linked Immunosorbent Assay 5
Immunochromatographic Lateral Flow Test Strips 5
Polymerase Chain Reaction 5
DNA Hybridization 6
Factors Affecting Pathogen Contamination 7
Type of microorganism 7
Temperature and Time 9
Moisture and Water Activity 11
Chemical Addition 13
Surface Area 14
Matrix 16
Protein 16
Carbohydrates 18
Fats 19
Minerals 20
Conclusion 21
References 22
Figures 31
Chapter 2 - Identifying Sources of *Salmonella* Contamination in Animal Food and Pet Food Manufacturing Facilities 32

Summary 32
Introduction 33
Materials and Methods 34
Facilities and Swabbing. 34
Qualitative Salmonella analysis of swabs. 36
Statistical Analysis 37
Results and Discussion 37
References 40
Tables 43
Chapter 3 - Effects of a Dry Acidulant Coating to Prevent *Salmonella* Contamination in Dry Extruded Pet Foods 50
Summary 50
Introduction 52
Materials and Methods 53
Kibble Production and Characterization. 53
Preparation of Coated Samples. 54
Preparation of Inoculum. 54
Inoculation and Storage of Samples. 55
Sample Plating and Enumeration. 56
Palatability Testing (Exp. 3) 57
Diet 57
Kibble Coating. 57
Palatability Testing. 58
Statistical Analysis. 58
Results and Discussion 59
References 63
Tables 65
Chapter 4 - Effects of a dry acidulant addition to prevent *Salmonella* Contamination in Poultry Feed 76
Summary 76
Introduction 77

Materials and Methods 78
SBS Inclusion 78
Salmonella Inoculum Preparation and Inoculation. 79
Sample Plating and Enumeration. 79
Statistical Analysis. 80
Results and Discussion 80
References 83
Figures and Tables 86

List of Figures

Figure 1.1 Chemical structure of sodium bisulfate (SBS). 31
Figure 2.1. Number of swabs collected per facility that were tested for presence of *Salmonella* 48
Figure 2.2. Percentage of swabs per facility testing positive for *Salmonella*. 49
Figure 3.1. Effect of sodium bisulfate (SBS) coating on *Salmonella* contamination in commercial dog food (Experiment 1). 73
Figure 3.2. Effect of sodium bisulfate inclusion level on *Salmonella* concentration in commercial cat food (Experiment 1). 74
Figure 3.3. Effects of sodium bisulfate inclusion level on salmonella concentration in dog food prior to enrobing (Experiment 2). 75
Figure 4.1. Effect of dry acidulant inclusion level on *Salmonella* Enteritidis level[1, 2] 87
Figure 4.2. Effect of day on *Salmonella* Enteritidis level[1, 2] 88
Figure 4.3. Effects of dry acidulant inclusion level on *Salmonella* Enteritidis over time[1] 89

List of Tables

Table 2.1. Main effect of facility on *Salmonella* contamination. 43
Table 2.2. Sampling locations in each facility 44
Table 2.3. Main effect of surface type on *Salmonella* contamination 46
Table 2.4. Nesting of surface type within facility on *Salmonella* contamination. 47
Table 3.1 Exp. 1. Effects of sodium bisulfate (SBS) coating concentration in commercially-produced cat or dog food on *Salmonella* concentration over time[1] 65
Table 3.2. Exp. 1. Effects of cat or dog food type on resultant *Salmonella* concentration and product characteristics[1] 66
Table 3.3. Exp. 2. Effects of sodium bisulfate (SBS) coating concentration in dry extruded dog food on *Salmonella* concentration over time[1] 67
Table 3.4. Exp. 2. Effects of dog food type on resultant *Salmonella* concentration and product characteristics[1] 68
Table 3.5. Exp. 2. Correlation among *Salmonella* concentration, ... 69
Table 3.6. Palatability of Dog Food Coated with Sodium Bisulfate 70
Table 4.1. Formulation and ingredient list[1] of the all-flock mash 86

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Chapter 1 - Interaction between Surface Area and Pathogen Contamination in Animal Food

Introduction

Food for both humans and animals may be a potential vector for bacterial and viral pathogens. Foodborne pathogens lead to an estimated 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths each year (Scallan et al., 2011). Notably, the numbers referenced do not include every case of foodborne illness, as many cases are presumed to go unreported. It is possible for pathogens to contaminate any type of food, but physical and chemical characteristics sometimes make one type of food a better pathogen vector than another. A vector is an organism that has the ability to transmit a disease from a plant or animal to another. Some examples of foods that have been commonly associated with pathogen contamination in the past include peanuts, tomatoes, cereal grains, milk, seafood, meat, and pet food.

Of the many types of pathogens that may cause illness, a select few are responsible for the majority of recent illnesses, hospitalizations, and deaths. In the past decade, norovirus caused the most illnesses; nontyphoidal *Salmonella* spp., norovirus, *Campylobacter* spp., and *T. gondii* caused the most hospitalizations; and nontyphoidal *Salmonella* spp., *T. gondii*, *L. monocytogenes*, and norovirus caused the most deaths. *E. coli* was also responsible for some illnesses, but mostly those related to meat products (Ge et al., 2013).

In addition to human foodstuffs, there have also been many recalls of animal food due to adulteration with pathogens. There are diverse populations of *Salmonella*, *E. coli*, and

Enterococcus in animal food, and recent research points to animal food potentially serving as a vector for porcine epidemic diarrhea virus (Dee et al., 2014). Of these pathogens, *Salmonella* contamination is most frequently associated with recalls, as it may be present in both animal feed (Ge et al., 2013) and pet food (KuKanich, 2011). In fact, *Salmonella* was responsible for 40% of the total amount of animal food entries of the past 4 reportable food registry annual reports (FDA RFR, 2015). *Salmonella* can cause Salmonellosis, a gastrointestinal illness.

In 2012, there was an outbreak of human *Salmonellosis* related to contamination of dog food (CDC, 2012). From April to July 2012, there were 49 reported cases of human infections with the outbreak strain of *Salmonella*. Among 24 patients with available information, 42% were hospitalized, and no deaths were reported. After interviews had been conducted, 22 of 28 (79%) ill persons reported contact with a dog in the week before becoming ill, and 11 of 28 (39%) ill persons identified a dry dog food produced by Diamond Pet Foods that was produced at a single manufacturing facility in South Carolina. The *Salmonella* serotype identified in the ill persons was the same as that present in an unopened bag of dry dog food produced by Diamond Pet Foods at this facility. The outbreak resulted in a recall of 17 brands representing more than 30,000 tons of dry dog and cat food produced at the facility. While this major recall is just one example of a biological pathogen causing illness, it was one of the first times when a hazard associated with animal food became a human health concern. It is important to understand the mechanisms of pathogen contamination and their potential impacts on human and animal health. This understanding may help lead to improved strategies to minimize or prevent hazards in animal food. Ultimately, this improves animal health and may decrease human foodborne illness (Medina, 2004).

Methods of Detecting Pathogens in Animal Food

There are numerous methods that may be employed to measure pathogenic contamination; the appropriate method depends upon the circumstances of the situation. For example, the range of methods used to detect molds extends from simple scanning with an ultraviolet light to extensive high performance liquid chromatography. Recent advances in biological hazard detection include examples where *Listeria monocytogenes* is detected by optical density using a spectrophotometer (Sirsat et al., 2014), and Surface Plasmon Resonance (SPR) biosensors used for other food pathogen detection (Yakes et al., 2013). Some methods are discussed below that may be used to detect *Salmonella* include: culture onto selective media, immunomagnetic separation, enzyme immunoassay, enzyme-linked immunosorbent assay, immunochromatographic lateral flow test strips, polymerase chain reaction, and DNA hybridization.

Culture onto Selective Media

Culture onto selective media is a method of *Salmonella* detection that is commonly used today. Some selective media can include modified semisolid rappaport-vassiliadis (MSRV) and tetrathionate (TT) broth. This method's accuracy may depend on the choice of selective media (Soria et al., 2011). The study by Soria et al. (2011) compared two selective culture methods and polymerase chain reaction (PCR), which also demonstrated that incubation time influenced the accuracy, whereas samples that were incubated longer resulted in more *Salmonella* positive samples. Growth of other non-*Salmonella* bacteria may also interfere with the results by decreasing the likelihood of obtaining well isolated colonies. Overall, selective media is an

accurate technique that is widely used today; however, because of the length of time to achieve results, it may be undesirable to some.

Immunomagnetic Separation

Immunomagnetic separation is widely used to rapidly isolate and concentrate food pathogens for the diagnostic methods, such as PCR and immunoassays (Shim et al., 2013). When combined with fluorescent and gold nanoparticles, it can be used to rapidly detect food pathogens. This method offers many advantages, such as simplicity, ultrasensitivity, easy to perform, and results can be obtained within a short amount of time, 45 minutes. One thing that could potentially have a negative impact, and therefore must be taken into consideration, is obtaining the correct antibodies for the specific type of pathogen being detected. Overall, immunomagnetic separation appears to be a simple, quick, accurate method of bacteria detection, which is highly desired by the pet food industry today.

Enzyme Immunoassay

Enzyme immunoassays generally involve labeling an antibody or antigen with an enzyme and then measuring enzyme activity inhibition. It can be completed within hours to ensure early detection, and has a high sensitivity. However, a study done by Leon-Velarde et al. (2009) suggests that enzyme immunoassays do not always have a high sensitivity. A combination of enzyme immunoassay along with immunomagnetic separation was studied within a single test format. The detection level was similar to that of other conventional methods, and results can be obtained within 48 hours, and minimal laboratory equipment is required. The enzyme

immunoassay, and potentially combined with immunomagnic separation, provides a promising method of *Salmonella* detection at the farm level.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) provides an antibody-based approach in which results can be obtained in a much shorter time and have a higher sensitivity than standard selective culture methods (Park et al., 2014). While there are several advantages to ELISA, the sensitivities of different commercial ELISAs can differ, leading to possible false negative results.

Immunochromatographic Lateral Flow Test Strips

Immunochromatographic lateral flow test strip assays are popular among diagnostic techniques used to detect bacteria, such as *Salmonella* spp., *Listeria* spp., and *Escherichia coli*. One disadvantage of this test method is its low sensitivity with a detection limit of $10^6 – 10^8$ cells/ml, which may be insufficient for use in some applications. Because of this, it may not be the best method for rapid and accurate detection of foodborne pathogens, and other methods should be used until improvements to immonuchromatographic lateral flow test strips are achieved.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) may be used for the quantitative detection of specific bacteria, as well as for their confirmation and serotypes (Hein et al., 2006; Jacobsen et al., 2007. A method commonly used today to determine the concentration of a pathogen is real-time quantitative PCR (RT-qPCR). The concentration of bacteria is inversely related to the

number of cycles of PCR (Cusick et al., 2015), so a higher concentration of bacteria requires fewer cycles of the PCR in order to detect genetic material. The use of specific primers allows one to further determine which serotype may be detected. PCR is regarded as a powerful technique to detect food pathogens, but it does require purification of microorganisms by pre-cultivation, as well as numerous experimental steps to execute the procedure (Shim et al., 2013). The PCR analytical methods are highly sensitive and exhibit relatively fast turn-around time. These methods are becoming more desirable when compared to other methods due to their much quicker turn-around time, but may not be as precise as other more time consuming methods (Jacobsen et al., 2007).

DNA Hybridization

DNA hybridization uses specific gene sequences from the bacteria being tested as probes. Along with the use of PCR, DNA hybridization can be a quick and accurate method to testing for foodborne pathogens (Fitts et al., 1983). Using a relatively long target gene between two primers has been shown to add increased specificity in PCR-based detection of *Salmonella* in pure cultures (Jacobsen et al., 2007). Overnight enrichment is required, however, selective enrichment is not. One possible disadvantage to this method would be that specificity of some probes may differ, resulting in false negatives. For example, if one uses a very specific probe for a certain serotype of *Salmonella*, it may not detect all of the *Salmonella* cells present in the sample. Overall, DNA hybridization is a quick, simple, accurate method for use in today's industry.

There are multiple methods of pathogen detection, and the appropriate one to use depends upon the objectives for analysis: speed, accuracy, and price. Just as many factors influence which method of pathogen detection is appropriate, there are also multiple factors that impact the

magnitude of contamination in an animal food. Factors that may affect pathogen contamination are discussed next.

Factors Affecting Pathogen Contamination

Salmonella is a ubiquitous bacterium; it may be present everywhere. Because of the ubiquitous nature of bacteria, *Salmonella* has the ability to cycle through a host into the environment, back into another host, and back to animals through cross-contaminated food or water. *Salmonella* is a gram negative bacterium that has the ability to cause gastrointestinal illness in both humans and animals (CDC, 2012). There are a variety of factors that affect the likelihood of pathogen contamination. Some risk factors that may contribute to animal food and pet food contamination are poor sanitation practices, poor facility design or inadequate maintenance, lack of good manufacturing practices, poor ingredient control and handling, and poor pest control (Podolak et al., 2010). Sanitation can be described as the process of keeping facilities free from pathogens by implementing regular cleaning procedures of equipment surfaces and work areas. In addition to these risk factors, the type of microorganism, temperature and time, chemical addition, moisture and water activity, and surface area may also play an important role in determining the risk of contamination with bacterial contamination.

Type of microorganism

Pathogens thrive under specific environmental conditions, and there are different types of growth patterns specific to each type of pathogen. For example, in modified Welshimer's Broth (MWB), *Listeria monocytogenes* exhibits a bi-phasic growth pattern at room temperature, mono-phasic pattern at high temperatures (18°C), and no growth at low temperatures (4°C;

Tyrovouzis et al., 2014). In this example, MWB is used because it is specially formulated to allow for the growth of *Listeria monocytogenes* bacteria. Several attempts have been made to understand the growth pattern of *Salmonella* in different matrices. Due to its dependency upon water activity, temperature, pH, surface, and nutrient availability, there has not been one specific growth curve established for *Salmonella* (Kumar et al., 2015). In the study by Kumar et al. (2015), the growth pattern of *Salmonella* was examined on seafood surfaces. These are typically contaminated through cross-contamination, much like pet food. The *Salmonella* increased at temperatures higher than room temperature or remained stable at temperatures below room temperature from day 0 to day 1. Then decreased in a logarithmic manner thereafter, then stabilizing, but asymptotically not reaching zero colony forming units. The growth pattern of *Clostridium botulinum* is also dependent upon the environmental characteristics. *Clostridium botulinum* is a gram-positive spore-forming bacterium with the ability to produce the neurotoxin botulinum. In the beginning time points (t = 0 to 10 h), there is little growth during nutrient uptake. During the middle time points (t = 11 to 30 h), *C. botulinum* exponentially increases, and then decreases exponentially until a stable growth is achieved (Ihekwaba et al., 2015). The organism grows best when it is exposed to low oxygen conditions, such is in canned pet foods. A combination of high pressures and temperatures are used in the canning process to destroy *Clostridium botulinum* (USDA Food Safety Information, 2010). The type of microorganism impacts survival of *Salmonella* and *C. botulinum* due to its interaction with varying environmental conditions.

Temperature and Time

Temperature and time contribute to microbial lethality in animal food manufacturing (van Schothorst and Brooymans, 1982; Jones, 2011). This has been well established in pet food manufacturing, but may be increasingly important to consider in livestock feed production. Jones (2011) noted that both time and temperature are crucial in determining the quantity of *Salmonella* destroyed by the pelleting process. Previous researchers have suggested that temperatures during conditioning of pelleted feeds should reach 80º to 85ºC (Veldman et al., 1995; Jones and Richardson, 2004). In pelleted animal feeds, the risk of microbial growth can be lowered by holding the temperature within the recommended range, and providing adequate airflow to finished product to eliminate condensation during packaging and storage.

Dry pet food production typically uses an extrusion process to manufacture diets, rather than pelleting. There are some similarities between pelleting and extrusion. Extruded diets use more moisture, more shear pressure, and higher levels of expansion, which has an impact on the resulting food and microbial survival. Okelo et al. (2006) examined time and temperature combinations that were most likely to eliminate bacterial contamination. They evaluated *Bacillus stearothermophilus* spores as a surrogate organism for the impact of time and temperature on bacterial contamination because of its high heat tolerance compared to *Salmonella*. The finished product was also tested for presence of *Salmonella typhimurium*. The feed matrix was inoculated with the surrogate and extruded while simultaneously recording three key variables: extruder exit temperature (T), mash feed moisture content (M_c), and mean retention time of feed in the extruder barrel (R_t). When vegetative bacterial cells are subjected to adverse conditions, they may form dormant spores as a means of survival for months to even years (Beauchat et al., 2013). When the thermophilic surrogate was exposed to higher heat conditions than normal,

some cells likely formed spores. There was no combination of time, temperature and moisture content that resulted in a maximum amount of thermophilic bacteria spore killing. This suggests that there are other methods that should be researched to mitigate spores. However, there was a ridge of maximal spore killing indicated a reduction of 1.03 log cycles at T = 110ºC, M_c= 245 g/kg, and R_t = 11 s. A ridge of maximal spore killing refers to a specific range of time, temperature, and moisture content that results in generally the same amount of spore killing. No *Salmonella typhimurium* cells were recovered in the tested feed matrix post-extrusion at T = 83ºC, M_c = 285 g/kg, and R_t = 7 s. The combination of temperature and time agrees with the suggested pelleting temperatures recommended by Jones and Richardson (2004) and Veldman et al. (1995). Based on this work, it is recommended that thermal processing, whether by pelleting or extrusion, reaches a minimum temperature of 85ºC for non-thermophilic bacteria, and 110ºC for thermophilic bacteria for at least 11 s.

The combination of temperature and time are also important to consider post-processing as product is stored. Ideally, sanitation controls in the plant would prevent post-processing cross-contamination. This is particularly relevant because bacterial contamination in animal foods may grow or proliferate during storage if the environment is favorable and the food was contaminated. Pet foods are sometimes stored for up to two weeks at room temperature prior to shipment (Lambertini et al., 2015). During this time, pathogens, such as *Salmonella*, may adapt to changing environmental conditions (Podolak et al. 2010). For example, *Salmonella* typically grow in temperatures of 35º to 37ºC; however, they can adapt and thrive in temperatures near freezing (2ºC) and up to 54ºC (Beauchat, 2009).

Moisture and Water Activity

Traditionally, moisture and water activity (a_w) may influence pathogen growth in certain foods and feeds, and thus have been controlled in the manufacturing process to prevent pathogen growth and proliferation. Water activity is the amount of free water available for the growth of microorganisms. It should not be confused with the term moisture, which is the total amount of water, both free and bound, in a matrix. Low moisture is not always indicative of low water activity. For example, there have been instances where foods with low moisture, including peanut butter, chocolate, dried milk, and pet foods, have been linked to *Salmonella* contamination (Craven et al., 1975; Rushdy et al., 1998; Kirk et al., 2004; Smith et al., 2004; and Podolak et al., 2010). In many of these cases, the low moisture foods had greater water activity than intended. This suggests that there may need to be a change in process controls to better obtain the intended water activity. Control of water activity, particularly in low moisture foods like pet food and animal feed, is important to prevent bacterial growth. One way to control this is by limiting extraneous, or outside, moisture sources throughout the manufacturing facility. In animal food manufacturing facilities, certain moisture sources may be obvious, such as roof leaks or water addition by hoses during sanitation. However, animal food can also gain moisture through condensation or absorption via contact with humid air (Hinton, 2000; Hemmingsen et al., 2008; and Jones, 2011). Therefore, the drying and storage processes are important to managing water activity, and should be measured regularly in order to maintain control.

It is generally accepted that growth of pathogenic microorganisms is limited when water activity is below 0.85. This is because a food matrix stored in environmental conditions below the growth threshold prevents cells from surviving the initial osmotic shock phase and will lower or retard the survival from multipication to the death of the organisms. Due to changing

environemtnal conditions, *Salmonella* may adapt and form biofilms. Biofilms are formed when the environmental conditions are unfavorable to the growth of an organism and result in groups of cells adhering to a surface and becomes very hard to mitigate.

It is not clear whether *Salmonella* forms biofilms under low-moisture conditions (Solano et al., 2002). However, other confounding factors may impact the success of controlling water activity to minimize pathogenic bacteria growth. For example, when external storage conditions are above or below the range of typical growth conditions, *Salmonella* may be able to survive for months, or even years, in certain low-moisture foods (Beauchat, 2009). Janning et al. (1994) studied the survival of 18 different bacterial strains, including *Salmonella*, under dry conditions of a_w = 0.2 at 22ºC. Initially, there was a decreased bacteria count; however, *Salmonella* numbers remained stable for an extended period of time with sustained counts from 248 to more than 1,000 days to achieve a 1.0 log reduction.

While lowering water activity may help to reduce the growth of pathogenic bacteria, any bacteria that does grow may have greater heat resistance (Podolak et al., 2010). This is particularly relevant when drying occurs by dry heat compared to moist heat. Kirby and Davies (1990) air dried or moist dried pet foods contaminated with *Salmonella* to a_w less than 0.57 for 48 h. These results showed that the remaining bacteria in samples dried by dry heat had increased resistance to destruction when compared to bacteria prior to drying or those dried by moist heat. This theory was confirmed by Chiewchan et al. (2006), who dried rawhides to specific a_w levels via moist or dry heat and then measured resistance of remaining *Salmonella* bacteria to subsequent destruction. All samples dried by dry air had greater heat resistance than rawhides dried by moist heat.

Chemical Addition

The initial goal of animal food safety is to prevent hazard occurrence in the first place. However, there are occasions when it is either impossible or impractical to prevent adulteration by people, equipment, or ingredients. In these cases, proactive mitigation must control the hazard prior to its consumption by an animal. Proactive mitigation may be at a single point in time, such as thermal processing by extrusion or irradiation. While effective, this type of mitigation does not prevent potential post-processing cross-contamination. Additional control steps must be employed throughout the production process to prevent cross-contamination (Cochrane et al., 2015).

Chemical additives, either alone or in combination with a point-in-time mitigation strategy, have garnered great interest because their use may help prevent subsequent cross-contamination of pathogens in animal food. These chemical additives, including organic acids, formaldehyde, may act by reducing the water activity of the animal food, changing the pH, or other alteration that prevents subsequent pathogen growth and reduces active concentrations of the pathogen. Chemicals that have been used to control *Salmonella* in animal food have primarily consisted of blends of organic acids and formaldehyde (Smyser and Snoeyenbos, 1979; Ha et al., 2000; and Ricke, 2005). However, many of these chemicals may have harmful effects on the health of the workers in the animal food manufacturing facility, and can be corrosive to the production equipment. Furthermore, currently used chemicals are typically liquid, which may require specialized application equipment and are challenging to employ in a dry bulk solids system, such as animal food manufacturing.

A chemical that appears to be a practical alternative to those currently utilized is sodium bisulfate (SBS; $NaHSO_4$; Jones-Hamilton, Co.). Sodium bisulfate (Figure 1.1) is an acid-salt that

is present in a powdered form and is primarily used in the pH reduction in a variety of food matrices (Kassem et al., 2012). Sodium bisulfate is used in the poultry industry as a feed acidulant to reduce enzyme activity and is also used in the pet food industry to reduce struvite formation in cats (Kassem et al., 2012; Knueven, 2013). In the dairy industry it is used to reduce bacterial counts in bedding (Sun et al., 2008). Sodium bisulfate is hygroscopic; as moisture is absorbed, the compound dissociates into its sodium (Na^+), hydrogen (H^+), and sulfate (SO_4^{2-}) constituents (Sun et al., 2008).This dissolution of SBS in water leads to acidification to prevent further bacterial growth, in addition to a hygroscopic mechanism that draws water out of the cytoplasm, resulting in cell apoptosis. Cochrane et al. (2015, 2016) determined that SBS inclusion in both poultry feeds and pet food ingredients, including poultry by-product meal and meat and bone meal, was effective at reducing bacterial contamination. Clearly, SBS as a chemical additives may be effective at controlling biological hazards; but its effectiveness could be dependent upon other factors.

Surface Area

There has been little research done to evaluate the effect of surface area on pathogenic microorganism contamination. In dry pet food, it is typical for cat food kibbles to be smaller in size than dog kibbles, thus resulting in a larger surface area to volume ratio in cat foods. It is currently unknown how surface area or its relation to volume may impact the growth of pathogenic bacteria. Phumgamngoen et al. (2011) described that topographical features of foods can facilitate entrapment and attachment of microbial cells. In dry pet food, the surface can appear to be rather smooth visually. However, extrusion actually produces a surface with numerous valleys on the surface that lead to a large surface area that may facilitate bacterial

attachment (Phumgamngoen et al., 2011). For example, Phumgamngoen et al. (2011) found matrices with greater surface area to volume ratios resulted in about a 1.0 log increase in the *Salmonella* contamination compared to the samples with a lower surface area to volume ratio. *Salmonella* present on the more topographical feature-heavy surfaces also experienced a greater level of resistance to heat (Phumgamngoen et al., 2011). According to these results, it may be hypothesized that dry extruded pet food kibbles with a lower surface area are potentially less likely to harbor bacterial attachment and growth than those with rougher surfaces that have a higher surface area per kibble.

Not only might surface area impact pathogen contamination, but also the quantity of dry chemical required to effectively coat the product for pathogen reduction. In products where a chemical additive is applied to help reduce or eliminate a pathogen, a larger surface area may require a greater quantity of the additive to achieve the same efficacy as a product with a smaller surface area. Thus, the determination of surface area is important to evaluate treatment efficacy.

One method to determine surface area is the Brunauer, Emmett, and Teller method. The BET method uses the amount of gas adsorbed by the surface. The surface area can then be calculated using the adsorption isotherm by means of the BET equation. There are other conventional methods that can be used to determine the amount of surface area, including the use of calipers to measure height and width. From these measurements, the surface area can be calculated according to the surface area equation specific to the shape of the particle being measured. For example, a cylindrical shaped particle requires the measurement of thickness (height) and the diameter. Using these parameters, the surface area can be calculated as: surface area = (height × circumference) + ($2\pi r^2$). A drawback of caliper measurement is that it only measures the outside surface, and does not account for internal surfaces or divots in the product.

Thus, the caliper measurement may not be as accurate as the BET method. These are just two examples of surface area measurement, but they demonstrate that the appropriate method depends upon the purpose and its requirement for accuracy and precision.

Matrix

While the type of microorganism, temperature and time, moisture and water activity, chemical addition, and surface area all impact the magnitude of pathogen contamination, it is their relationship with the pet food matrix that impacts these other factors the most. Specifically, the makeup of the protein, carbohydrate, fats, and mineral fractions within a matrix can have a substantial impact on the quantity and pathogenicity of bacterial contamination.

Protein

One of the largest components of pet food is protein, composing 20 to 30% of the dry matter of a pet food. Protein, as an ingredient, is a large component of a pet food, consisting of multiple essential and nonessential amino acids (Yamka et al., 2007; Laflamme, 2008). Because of the energy available from protein, foods with higher protein content may have the ability to harbor greater amounts of bacterial growth.

There is limited research on the effect of protein matrix in pet food and the viability of pathogenic microorganisms. However, the viability of pathogenic microorganisms in protein of human foods, especially peanut butter, has been reported. There have been several outbreaks associated with peanut butter and *Salmonella* contamination. One outbreak that occurred in 2009 resulted in over 700 illnesses throughout the United States due to contamination of peanut butter with *Salmonella* Typhimurium (CDC, 2009). Burnett et al. (2000) utilized research regarding

peanut butter to extrapolate the data and suggest that *Salmonella* is likely to survive for prolonged periods of time in storage if it is in a protein-based matrix.

One of the main sources of protein in companion animal diets is meat due to its crude protein content (21%) and balance of amino acids (Thompson, 2008). Some examples of protein sources used in pet food are poultry byproduct meal, corn gluten meal, meat and bone meal, and soybean meal, with a limited contribution of protein from grains, such as whole corn, whole wheat, barley, and rice (Thompson, 2008).

There has been previous research performed to study bacterial contamination in raw meats, which are high in protein compared to plant sources. Both pathogenic and spoilage microorganisms may be transferred to raw meat from the outer surface of an animal carcass via fomites and rinses (Zulfakar et al., 2013). Fomites are inanimate objects or substances that hav the capability to transmit infections organisms from one individual to another. Medina (2004) described that bacteria readily attach to meat surfaces, but are not easily removed. For example, *Salmonella* cells entrapped in the ridges and crevices of poultry muscle have been shown to be present even after 40 washes (Lillard, 1989).

The role of bacterial attachment is particularly problematic in raw meat pet diets, which are becoming increasingly popular in the United States. Screening raw meat diets for pathogenic microorganisms, including *Salmonella* is important for the health of both humans and animals because there is no thermal kill step to eliminate bacteria prior to feeding to animals, and direct human contact.

The role of proteins, as a nutrient, to preferentially proliferate bacteria is not restricted to only animal-based proteins. In a study performed by Ge et al. (2013), a multitude of feed ingredient samples were collected and tested for the occurrence and antimicrobial susceptibility

of *Salmonella, Campylobacter, Escherichia coli,* and *Enterococcus*. No samples harbored *Campylobacter* or *E. coli* 0157:H7; but *Salmonella, E. coli,* and *Enterococcus* were present in 22.9%, 39.3%, and 86.6% of the samples, respectively. The samples tested were categorized by animal-derived ingredients or plant-derived ingredients. All of the animal-derived ingredients tested positive for *Salmonella, E. coli,* and *Enterococcus*. Of the plant-derived ingredients, only oilseed byproducts, soybean meal, and cottonseed meal tested positive for *Salmonella,* but almost all of the plant- derived ingredients tested positive for *E. coli* and *Enterococcus*. This raises a question as to the role of physical or chemical characteristics of animal- and plant-based protein sources and its impact on bacterial proliferation. Regardless, it is evident that bacteria, especially *Salmonella* survives in protein-containing ingredients, and the type of protein may impact its growth and viability.

Carbohydrates

Another major nutritional component of animal food are carbohydrates. These are a broad category of compounds made from polysaccharides that include starches, oligosaccharides, and celluloses (Thompson, 2008). Carbohydrates supply glucose for cellular energy, as well as also provide fiber in pet diets. Typical sources of carbohydrates in pet foods include, but are not limited to, various grain flours, brown rice, oats, sorghum, and potatoes (Thompson, 2008). Sources of fiber include wheat bran, rice bran, soybean hulls, beet pulp, powdered cellulose, chicory root, inulin, and fructooligosaccharides (Thompson, 2008). There is limited data regarding the role of carbohydrates or their composition and its effect on bacterial contamination in either animal or human foods. Therefore, it may be suspected that other nutritional

components are responsible for most of the variability in matrix-dependent differences in bacterial contamination.

Fats

Fats are another macronutrient that is present in pet food. The mixture of fatty acids in pet diets provides the essential unsaturated fatty acid, linoleic acid, and omega-3 fatty acids (e.g. eicosapentaenoic acid and docosahexaenoic acid; Thompson, 2008). Most added fat included in pet diets comes from animal-derived sources, which is produced via the rendering process. Other commonly used sources of added fat are vegetable oils that are rich in linoleic acid or fish oil and flaxseed oil for omega-3 fatty acids (Thompson, 2008). Other sources of fat in a pet food may come from other ingredients, such as protein meals and grains.

Podolak et al. (2010) described that *Salmonella* can survive for extended periods of time in foods with a high fat content, like peanut butter. Traditionally, added fat is applied after extrusion. Thus, any bacterial contamination in these ingredients is problematic because it is applied after the thermal kill step in dry extruded pet foods. It is therefore pertinent to ensure fat does not have *Salmonella* or other bacteria during receiving, and care should be taken during storage to prevent proliferation of existing bacteria. The degree of survival may depend on relative humidity and atmospheric storage conditions of the fat. Chemical additives may be a desirable alternative to eliminate and prevent growth of bacteria during storage and prior to application to pet foods.

Minerals

The AAFCO nutrient profiles contain 12 essential minerals for dogs and cats, usually added to a diet in the form of a premix. These ingredients are added in very small amounts of each, but can account for nearly half of the individual ingredients in a pet food (Thompson, 2008). Two minerals have been suggested to potentially reduce pathogenic bacteria: iron and sodium.

Iron is essential to virtually all living organisms, and in the form of heme, is vital to numerous metabolic functions, especially oxygen transportation via hemoglobin. In animals, iron must be present in sufficient amounts in the diet; otherwise, a myriad of health problems could occur, including anemia (Naigamwalla et al., 2012).

Bacterial growth also depends on the availability of iron (Crosa, 1989). One of the most common occurring activities of microorganisms is the production of siderophores, or low-molecular weight iron chelators that have very high constants of association for their complexes with iron (Crosa, 1989). The four types of siderophores produced by microorganisms, and their chemical structures are listed in Figure 1.2. There are four iron uptake systems in *E. coli* that use siderophores produced by *E. coli* to produce siderophore-iron complexes, which are uptaken by outer membrane receptor proteins, and are used in the enterobactin and aerobactin systems (Figure 1.3) to synthesize new bacterial cells, resulting in increased bacterial growth. In this pathway, ferrous (Fe^{2+}) is used, which is also the form of iron used to transport hemoglobin. Because iron is an important mineral in animal food, it is important to reduce or eliminate bacterial growth, as to not reduce the amount of iron in the diet.

Another macromineral, sodium, may play a role in the inhibition of bacterial growth; however, little research has been performed to prove this. It is thought that by including a higher

concentration of sodium chloride may inhibit bacterial growth by limiting osmotic action. The sodium draws water out of the bacteria cell cytoplasm, resulting in cell death. Interaction with other minerals have not been studied in pet foods. Due to the fact that there are numerous minerals included in an animal food diet, it is important to implement other measures to inhibit or mitigate the growth of pathogenic microorganisms to prevent nutrient losses that may result in severe health issues in the animal.

Conclusion

Bacterial contamination of animal food and animal food ingredients has been reported since the early 1950's, and continues to be a concern (Harris et al., 1997). Over the years, *Salmonella* contamination in animal food ingredients has been substantially reduced; however, there are still millions of cases of salmonellosis in both the United States and other industrialized nations (Jones, 2011). *Salmonella* contamination in animal food production facilities is a growing concern. The bacteria has been the cause of 40% of pet food recalls in the past 5 years, and there are potential human health implications because pet food is a direct human contact food. There are numerous methods that can be used to detect *Salmonella* in various animal food ingredients and animal foods, but it is still not well understood what surfaces are more likely to cause proliferation in animal food manufacturing facilities. In addition, there are many factors that determine a food's susceptibility to pathogenic microorganisms, including the type of microorganisms, temperature and time, moisture and water activity, chemical addition, surface area, and animal food matrix. A potential method to reduce *Salmonella* contamination in pet food is through the use of acidifiers and desiccants to destroy and inhibit growth of bacteria. The objective of this thesis was to quantify *Salmonella* contamination in livestock feed and pet food

manufacturing facilities, and propose mitigation measures to mitigate the pathogen's presence in animal food.

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Figures

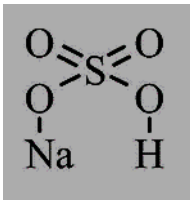

Figure 1.1 Chemical structure of sodium bisulfate (SBS).

Chapter 2 - Identifying Sources of *Salmonella* Contamination in Animal Food and Pet Food Manufacturing Facilities

Summary

Salmonella contamination in animal food, including livestock feed and pet food production facilities is a growing concern. *Salmonella* is a potential biological hazard in animal food, and may contaminate livestock feed and pet food through ingredients or cross-contamination at manufacturing facilities. There is zero tolerance of *Salmonella* of any serotype in pet food, while the zero tolerance in other animal food is for serovars pathogenic to those specific species. The prevention of cross-contamination may require sanitization of animal food manufacturing surfaces, but there is limited information regarding the natural presence of bacteria on different types of surfaces or within different facilities. The objective of this study was to investigate sources of *Salmonella* contamination throughout livestock feed and pet food manufacturing facilities, which may lead to cross-contamination of animal food with the bacteria.

Contamination was monitored through the collection of surface swabs from various equipment and environmental locations from two livestock feed mills and two pet food manufacturing faciliteis on a single sampling day. Collected surface samples were from a variety of equipment and structural surfaces, including concrete, dust, plastic, rubber, and broom bristles. All collected swabs were qualitatively analyzed for *Salmonella* according to the FDA Bacteriological Analytical Manual (BAM). Data were categorized by facility (A to D), type (equipment or structural), and surface (concrete, metal, plastic, rubber, or dust), and analyzed using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC). Facility A, a livestock feed mill, had more than twice the positive locations than facility B, C, or D. Overall, swabs collected

from rubber and plastic surfaces were more likely to be *Salmonella*-positive compared to swabs collected from concrete, dust, or metal ($P < 0.01$). The main effects of facility, sample location, and their interaction were different ($P < 0.03$, 0.01, 0.002, respectively). Presumptive *Salmonella*-positive swabs were further serotyped, with several serovars identified as Enteritidis, Havana, Typhimurium var 5, (4,12 i), and Agama. Based on these results, surface type and location should be taken into consideration when controlling *Salmonella* contamination. Particular emphasis should be placed on rubber and plastic surfaces to prevent cross-contamination into animal food.

Introduction

Salmonella is a gram negative bacterium that is an important cause of human gastrointestinal illness (Heymann, 2008). Each year, *Salmonella* is estimated to cause one million illnesses in the United States, with 19,000 hospitalizations and 380 deaths (CDC, 2015). Animal food has been linked to *Salmonella* transmission, and therefore should be considered when creating control programs to address bacterial contamination in a food safety system (EFSA, 2010). Animal food includes foods for livestock, poultry, equine, and pets.

Contamination of animal food with *Salmonella* may pose a risk of infection to animals, which may then be potentially transmitted to the human food chain during colonized animal harvest (Crump et al., 2002). Ingredients and humans may serve as potential vehicles of *Salmonella*, which may then colonize with biofilms in animal food manufacturing facilities and lead to post-processing cross-contamination (Davies and Wales, 2010; Wierup and Haggblom, 2010; Torres et al., 2011; and Seiferth et al., 2013).

Research has shown that manufacturing animal food that is contaminated with the *Salmonella* surrogate, *Enterococcus faecium*, leads to substantial contamination throughout the processing facility (Huss et al., 2015). While previous research describes the role of bacterial contamination on equipment and surfaces, it is limited to those for livestock and poultry feed mills (Pellegrini et al., 2014; Davies and Wales, 2010; Torres et al., 2011; Whyte et al., 2002). The objective of this study was to investigate sources of *Salmonella* contamination from various equipment and environmental locations found in pet food manufacturing facilities on a specific sampling day and compare them to those in livestock feed mills.

Materials and Methods

Facilities and Swabbing. A total of four livestock feed (n = 2) and pet food (n = 2) manufacturing facilities located within the Midwest region of the United States were selected for one-time sampling. At each facility, up to 40 environmental swabs were collected (Table 2.1), with number of swabs collected from each facility based on the availability of the equipment and step in manufacturing. All surface samples were collected using sterile, commercially prepared swabs containing buffered peptone water (Puritan Medical Products Company LLC, Gilford, ME). At each facility, swabs were collected from a variety of surfaces, including equipment, concrete, dust, metal, plastic, and rubber.

Facility A. Facility A was a livestock feed manufacturing facility that manufactured primarily swine and beef cattle diets on a daily basis that were packaged in sacks, stored on palettes. Swabs were collected from a variety of locations, including the roller mill, bagger discharge, conveyor belt for bagger machine, receiving pit grate, ingredient receiving, interior wall of

bucket elevator, worker boot bottom, receiving leg bucket and belt, interior and belt of roller mill, floor dust in feed manufacturing areas, broom bristles in raw material receiving, and warehouse floor.

Facility B. Facility B was a pet food manufacturing plant that manufactured both custom diets for a specific label and generic diets for dogs and cats. Swabs were collected from a variety of locations, including flooring in the warehouse and areas around production equipment, ingredient pit grating in the receiving area, broom bristles from broom in receiving area, fat intake inlet, floor dust in receiving area, ingredient bucket elevator boot, floor dust in feed manufacturing area, finished product bin at packaging discharge, mixer ribbon, interior wall of pellet mill cooler, exterior of pellet mill bottom, extruder die plate, conditioning discharge, post-pellet mixer liquid application nozzle, hammermill hammers and magnets, worker shoes, and dust in warehouse area.

Facility C. Facility C was a livestock feed manufacturing facility and produced livestock diets for all species. Swabs were collected from a variety of locations, including the exterior of pellet chillers and dust around pellet coolers, dust on mixer, floor dust, wall grate and door threshold of mixer and pellet cooler room, corn receiving pit grate, floor dust by receiving pit grate, exterior and interior hammermill and roller mill, floor dust of grinding room, hammermill screen, hammermill dust, area in front of mouse trap, major and minor scales, inside of pellet mills, top of micro-ingredient bins, broom bristles, worker shoes, liquid receiving area, micro-ingredient bins, and bucket and belt of mash leg.

Facility D. Facility D was a pet food manufacturing facility. Swabs were collected from a variety of locations, including receiving area, floor dust, grain pile at receiving surge, top of dust collection system, control room floor, agitator column, grain receiving area, exterior of hammermill, exterior of mixer, receiving pit, aggregate on floor by receiving floor, ingredient room plastic shovel, exterior of receiving leg, aggregate by receiving leg, worker shoes, broom bristles, and scales.

Qualitative Salmonella analysis of swabs. Swabs were qualitatively analyzed following the FDA's Bacteriologial Analytical Manual (BAM), with some modifications. After swab collection was completed, all swabs were transported on ice to the Grain and Feed Microbiology and Toxicology Laboratory (Kansas State University, Manhattan, KS) for immediate analysis. For pre-enrichment, collected swabs were incubated at 35ºC ± 2°C for 24 hours. After pre-enrichment, swabs were vortexed vigorously, with 1 mL of liquid transferred to 9 ml of tetrathionate (TT; Difco, Franklin Lake, NJ) broth and 0.1 mL of liquid transferred to 10 mL of Rappaport-Vassiliadis (RV; Difco, Franklin Lake, NJ) broth. Inoculated TT and RV broths were incubated at 42ºC ± 2°C for 24 hours for selective enrichment. From the selectively enriched broths, 10 µL from each broth was streaked on to Xylose Lysine Desoxycholate (XLD; Difco, Franklin Lake, NJ) agar and incubated at 35ºC ± 2°C for 24 hours. From XLD, colonies typical of *Salmonella*, pink with or without black centers, and atypical colonies, yellow with black centers, were inoculated to a lysine iron agar (LIA; Difco, Franklin Lake, NJ) slant, a triple sugar iron (TSI; Difco, Franklin Lake, NJ) agar slant, and streaked to a trypticase soy agar (TSA; Difco, Franklin Lake, NJ) plate for subsequent serotyping. Inoculated biochemical assays were incubated at 35°C ± 2°C for 24 hours. Colonies on TSA were stored at 4ºC until serotyping after

incubation. Based on LIA and TSI reactions, presumptive positive isolates were transferred to nutrient agar slants (NA; Difco, Franklin Lake, NJ) from previously inoculated TSA and incubated at 35ºC ± 2°C for 24 hours for serotyping. Inoculated NA slants were sent to the National Veterinary Services Lab (NVSL; Ames, IA) for serotyping.

Statistical Analysis. Data were analyzed using the GLIMMIX procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC) with the fixed effects of facility (A to D), surface (concrete, metal, dust, plastic, or rubber), and their interactions. The main effects considered were facility (A, B, C, D), and surface type nested within facility. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

The type of manufacturing facility, surface type, and their interaction were all related ($P < 0.05$) to the level of *Salmonella* contamination (Tables 2.1, 2.2, 2.3, and 2.4, respectively). Specifically, Facility A had a greater ($P < 0.05$) percentage of positive *Salmonella* samples than the other three facilities (38.1 vs. 10.5, 12.5, and 10.7 for Facility A vs. Facilities B, C, and D, respectively; Table 2.1, Figure 2.2). Surface type also impacted the percentage of positive *Salmonella* samples ($P < 0.05$; Table 2.3). More *Salmonella*-positive samples were collected from concrete (33.3%), plastic (30.0%) and rubber (44.4%) than metal (3.9%). These surface types were impacted by the type of facility in which they were found ($P < 0.05$). For example, 100% and 44.4% of the concrete surfaces swabbed in the two livestock feed mills (Facilities A and C, respectively) were positive for *Salmonella*, while no concrete swabs were positive for the bacteria in the pet food manufacturing facilities (Facilities B and D). Dust appeared to be one of the primary locations associated with potential cross-contamination in pet food manufacturing

facilities. *Salmonella* was present in 33.3% and 18.2% of the dust samples from pet food manufacturing facilities (facilities B and D). These results agree with previous research which shows that floor dust generally is more likely to contain *Salmonella* than other surfaces (Whyte et al., 2002; Pellegrini et al., 2014). This could be due to the thorough cleaning of high traffic areas, but not practicing thorough cleaning procedures of the equipment.

Salmonella was present in 12.5, 0, 0, and 9.1% of metal samples from Facility A, B, C, and D, respectively. Facility A had 50% of the plastic surfaces test positive for *Salmonella*, but none of the bacteria was present on plastic in Facility C or D, and no plastic surfaces were swabbed in Facility B. Finally, rubber appeared to be a significant source of potential cross-contamination in three facilities as it had a 50% contamination rate in Facility A, B, and C, but 0% in Facility D. The higher rate of *Salmonella* contamination from rubber and plastic surfaces observed in this study may be due to the relatively rough nature of these surfaces in the sampled animal food manufacturing facilities. These rough surfaces are difficult to clean and may be a greater risk for *Salmonella* attachment. Previous research suggests that cleaning efficacy is surface dependent, and biofilms developed on rough material such as bricks and conveyors are the most resistant to cleaning agents (Somers and Wong 2004). After serotyping, several serovars of *Salmonella* were recovered, including Enteritidis, Havana, Typhimurium var 5, (4,12 i), and Agama. Of these, Typhimurium is known to form highly resistant biofilms on surfaces. Li et al. (2012) showed that of the top ten serovars of *Salmonella* found in humans, some of those same serovars were also found in the top 25 list of most common *Salmonella* serovars found in animal food. This suggests that illness caused by *Salmonella* in humans may originate from *Salmonella* contamination in animal food.

With these results, there are some limitations that should be addressed. There are over 5,000 animal food manufacturing facilities in the United States (FDA Registration Statistics, 2016). In this study, 2 animal feed manufacturing facilities and 2 pet food manufacturing facilities were sampled. While there was *Salmonella* found in all four facilities, there was not a large enough sample size to determine whether our findings can be true for all animal food manufacturing facilities in the United States. Thus, further research is needed to determine if our results can be extrapolated to all U.S. animal food manufacturing facilities.

In each facility there were varying numbers of samples and surface types that were collected (Table 2.2). In some facilities, there were limitations as to where we were able to sample. For example, one facility was not willing to shut down production for sampling purposes, limiting our swab collection to surfaces outside the manufacturing equipment. Alternatively, at another facility, we were able to sample inside and outside manufacturing equipment surfaces due to production shut down. In order to be able to specifically determine which surfaces are likely to harbor *Salmonella*, facilities should have equivalent number of samples from each surface type and facility.

In summary, these results suggest that there is substantial variability in the magnitude of *Salmonella* contamination across animal food manufacturing facilities. The livestock feed mills surveyed in this experiment had greater *Salmonella* contamination than the pet food manufacturing facilities, but it is most concerning that the bacteria was isolated from swabs collected from post-processing manufacturing equipment in a pet food facility. Concrete, plastic, and rubber surfaces were the most likely to be contaminated with *Salmonella* in this experiment. This information may be useful to develop targeted sanitization strategies for animal food manufacturing surfaces.

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Tables

Table 2.1. Main effect of facility on *Salmonella* contamination.

Facility	Positive/Total	*Salmonella, %*	SEM	*P* =
A	8/21	38.1[a]	7.37	0.03
B	2/19	10.5[b]		
C	5/40	12.5[b]		
D	3/28	10.7[b]		

[1]Samples were collected from four different facilities: 2 livestock feed manufacturing plants, and 2 pet food manufacturing plants.

[2]Samples were collected from five surface types: concrete, dust, metal, plastic, or rubber.

[a, b]Means within a column differ when $P < 0.05$.

Table 2.2. Sampling locations in each facility

	Facility			
sample location	A	B	C	D
bagger belt	✔			
bagging outlet	✔			
broom bristles	✔	✔	✔	
bucket elevator belt	✔		✔	
bucket elevator boot		✔		
concrete by mixer			✔	✔
concrete by hammermill				✔
concrete in manufacturing		✔		✔
concrete near receivimg	✔	✔		✔
conditioning discharge		✔		
door threshold in mixer room			✔	
extruder die plate		✔		
fat intake inlet		✔		
finished product bin		✔	✔	
floor of break room		✔		
floor of control room			✔	
floor of grinding room			✔	
floor of warehouse	✔	✔		
hammermill exterior			✔	✔
hammermill hammers		✔		
hammermill magnet		✔		
hammermill screen			✔	
hand agitator in receiving				✔
indoor fork lift				✔
interior of bucket elevator	✔		✔	
metal exterior of mixer			✔	✔
metal floor of grain receiving				✔
metal wall grate			✔	
mixer ribbon		✔		
pellet cooler exterior		✔		
pellet cooler interior		✔		
pellet mill exterior		✔	✔	
receiving leg bucket elevator	✔			
receiving pit grate	✔	✔	✔	✔
rodent trap			✔	
roller mill belt	✔			
roller mill interior	✔	✔	✔	

top of micro ingredient bins	✔	
trash dust in receiving room		✔
scale		✔
shovel (plastic)		✔

Table 2.3. Main effect of surface type on *Salmonella* contamination

Surface type	Positive/Total	*Salmonella*, %	SEM	*P* =
Concrete	5/15	33.3[a]	9.91	0.01
Dust	4/23	17.4[ab]		
Metal	2/51	3.9[b]		
Plastic	3/10	30.0[a]		
Rubber	4/9	44.4[a]		

[1]Samples were collected from four different facilities: 2 livestock feed manufacturing plants, and 2 pet food manufacturing plants.

[2]Samples were collected from five surface types: rubber, concrete, plastic, dust, or metal.

[a, b]Means within a column differ when $P < 0.05$.

Table 2.4. Nesting of surface type within facility on *Salmonella* contamination.

	Facility					
(positive/total) Item (%);	A	B	C	D	SEM	*P* =
Concrete	(1/1) 100.0[a]	(0/2) 0.0[bcd]	(4/9) 44.4[abc]	(0/3) 0.0[bcd]	18.15	0.002
Dust	(2/4) 50.0[abc]	(1/3) 33.3[abcd]	(0/7) 0.0[cd]	(2/11) 18.2[bcd]		
Metal	(1/8) 12.5[bcd]	(0/12) 0.0[cd]	(0/20) 0.0[cd]	(1/11) 9.1[bcd]		
Plastic	(3/6) 50.0[abc]	-	(0/2) 0.0[cd]	(0/2) 0.0[bcd]		
Rubber	(2/4) 50.0[abc]	(1/2) 50.0[abc]	(1/2) 50.0[abc]	(0/1) 0.0[cd]		

[1]Samples were collected from four different facilities: 2 livestock feed manufacturing plants, and 2 pet food manufacturing plants.

[2]Samples were collected from five surface types: rubber, concrete, plastic, dust, or metal.

[a, b, c, d]Means within a column differ when $P < 0.05$.

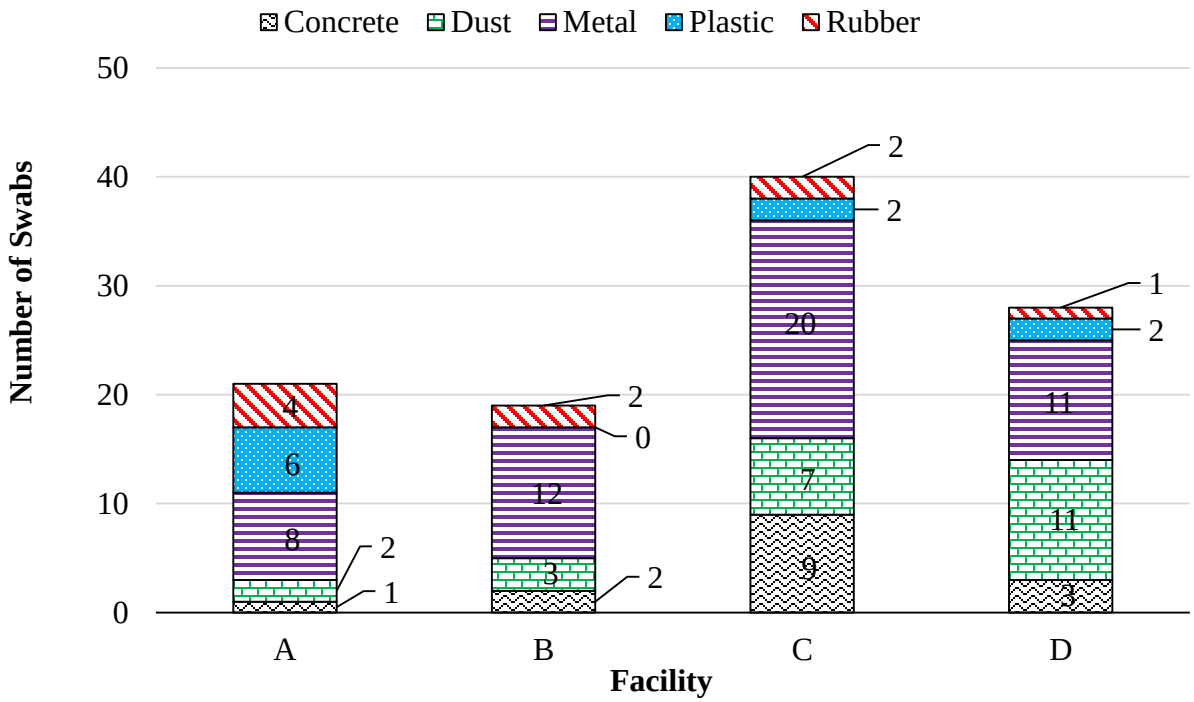

Figure 2.1. Number of swabs collected per facility that were tested for presence of *Salmonella*

[1]Samples were collected from four different facilities: 2 livestock feed manufacturing plants, and 2 pet food manufacturing plants.

[2]Samples were collected from five surface types: rubber, concrete, plastic, dust, or metal.

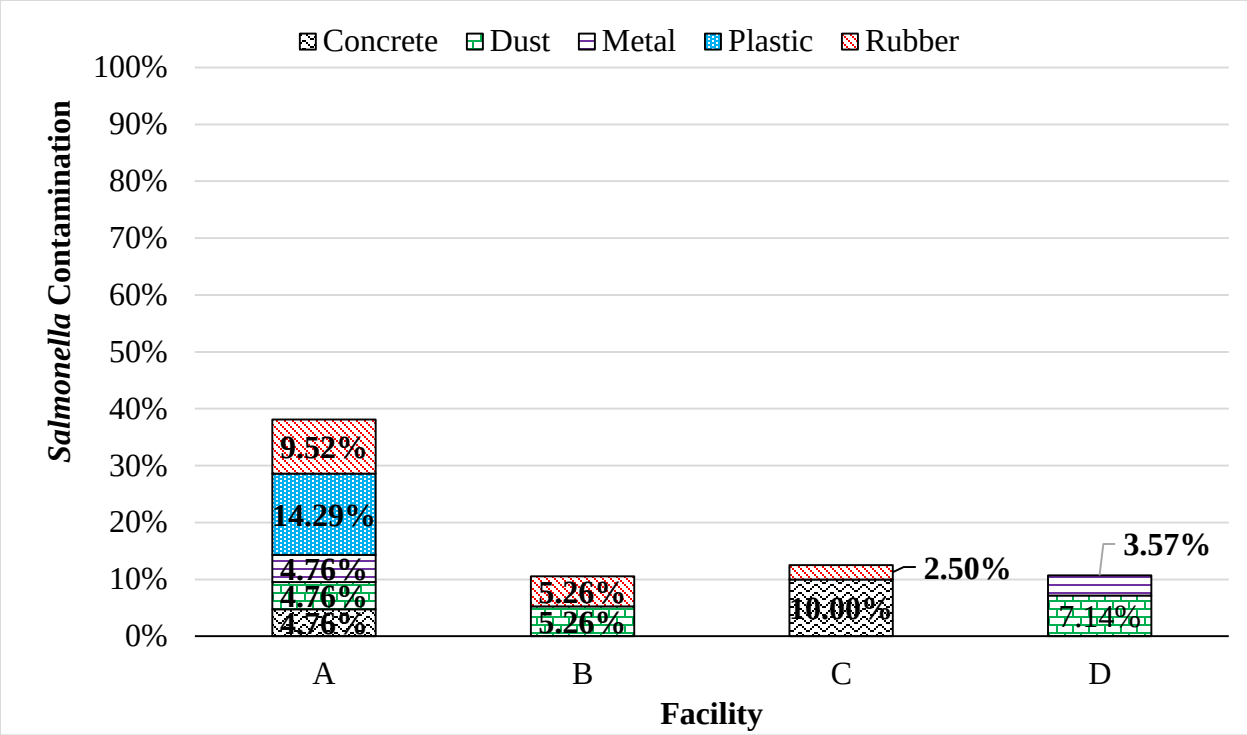

Figure 2.2. Percentage of swabs per facility testing positive for *Salmonella*.

[1]Samples were collected from four different facilities: 2 livestock feed manufacturing plants, and 2 pet food manufacturing plants.

[2]Samples were collected from five surface types: rubber, concrete, plastic, dust, or metal.

Chapter 3 - Effects of a Dry Acidulant Coating to Prevent *Salmonella* Contamination in Dry Extruded Pet Foods

Summary

With the implementation of the Food Safety Modernization Act and increasing consumer demands, animal feed safety is under increasing scrutiny. Particularly, *Salmonella* contamination has been identified as a potential biological pathogen of concern in the pet food industry, and has been the cause of several recent pet food recalls. One potential method to prevent or reduce *Salmonella* concentration in pet foods is through the use of acidifiers and desiccants to destroy and inhibit growth of bacteria. The objectives of these experiments were to determine if coating extruded pet food kibbles with a commercial powdered dry acidulant, sodium bisulfate (SBS; Jones-Hamilton, Co., Walbridge, OH, USA) would prevent and reduce *Salmonella* growth over time and to determine the impact of kibble type and size on the efficacy of SBS. In Exp. 1, 10 commercially extruded dry pet foods were utilized in a nested design with two pet food types: cat vs. dog, and three SBS concentrations within food type: 0, 0.6, and 0.8% for cat foods or 0, 0.2, or 0.4% for dog foods. In Exp. 2, a single formula of a custom-manufactured dry extruded dog food was utilized in a 4 × 3 factorial design with 4 kibble sizes (surface areas of 455, 997, 1,022, or 7,337 mm^2) and 3 SBS coating levels of SBS (0.0, 0.2, or 0.4%). In both experiments, kibbles

were analyzed for surface area, piece density, and bulk density. Next, kibbles were coated with varying levels of SBS and then inoculated with a *Salmonella* spp. cocktail (ATTC# 13076) on day 0, and analyzed for *Salmonella* on d 0, 1, 2, 7, and 14 by direct plating to xylose lysine deoxycholate (XLD) agar. In Exp. 1, piece density was correlated with *Salmonella* concentration ($P = 0.001$, correlation coefficient = 0.47), but not bulk density or surface area ($P = 0.16$ and 0.68, respectively). The main effects of pet food type, SBS concentration, and time, as well as their interactions, all significantly impacted *Salmonella* concentration ($P < 0.05$). By d 14, SBS coating resulted in more than a 2.1- and 1.2-log reduction ($P < 0.05$) in *Salmonella* concentration in cat and dog foods, respectively, compared to the uncoated kibbles. In Exp. 2, bulk density, piece density, and surface area were not correlated with *Salmonella* concentration ($P > 0.10$), and there was no effect ($P > 0.10$) of kibble size or the interactions, including kibble size on *Salmonella* concentration. However, coating kibble with 0.2 or 0.4% SBS resulted in a 2.0- or 1.6-log reduction ($P < 0.0001$) in *Salmonella* compared to uncoated kibble. Time also had a substantial effect on *Salmonella* concentration, and reduced the bacteria by 3.4-logs by 14 d ($P < 0.0001$). In conclusion, both time and the coating of kibble with a dry acidulant substantially reduce *Salmonella* concentration in the tested product. However, altering the bulk density and surface area of kibble in addition to application rate does not impact the efficacy of the acidulant coating.

Introduction

Salmonella is a gram negative bacteria that can cause a significant food safety-related illness in humans (Heymann, 2008; CDC, 2015). Because pet food is a direct human contact food, *Salmonella* contamination of these products may lead to human salmonellosis through cross-contamination in the home. For example, one large pet food recall in 2012 that was linked to a single manufacturing facility resulted in 49 cases of human salmonellosis, 24 of which required hospitalization (CDC, 2012). While this is an infrequent link, it is important to control *Salmonella* in pet foods for both human and animal food safety (Seiferth et al., 2015).

There are several options to control *Salmonella* contamination in animal food. The time × temperature combination reached during extrusion process should result in *Salmonella*-free kibble production, but does not prevent post-processing contamination. Chemical addition, such as commercial formaldehyde, medium chain fatty acids, organic acids, essential oils, and bacteriophage cocktails prior to or after extrusion has shown to help prevent post-processing contamination of kibble (Cochrane et al., 2015; Heyse et al., 2015). However, many of these chemicals may be hazardous for employees to apply, are not readily available, or may cause corrosion of manufacturing equipment. A potential alternative chemical to reduce post-processing *Salmonella* contamination is sodium bisulfate (SBS; Jones-Hamilton Co., Walbridge, OH). Sodium bisulfate is a weak acid in a bulk, dry, powdered form. Theoretically, the acidification action of SBS may reduce the pH on the exterior of kibble to prevent bacteria growth. Furthermore, the product has a desiccant action that may draw moisture from within the *Salmonella* cell membrane, reducing the viability of active bacterium. While the antimicrobial action of SBS is plausible, it is an attractive alternative to other chemical applications because it is already being utilized as an animal food preservative and silage additive in livestock feed, and

as an acidifier and preservative in pet foods (EFSA, 2014) with few detrimental effects to palatability, worker safety, or equipment surfaces. However, there has been little research evaluating SBS as a potential mitigating agent to prevent post-processing *Salmonella* contamination in pet foods. The use of SBS as a coating in pet foods may also present concerns associated with palatability. Therefore, the objectives of these three experiments were to evaluate 1) the ability of SBS-coated kibble to prevent and reduce on *Salmonella* growth over time, 2) the impact of kibble type and size on its efficacy, and 3) to determine the effect of SBS on the palatability of dry dog food.

Materials and Methods

Kibble Production and Characterization. In Experiment 1, 10 commercially-extruded pet foods (5 dog foods and 5 cat foods) were obtained from a third party supplier. In Exp. 2, a single diet was manufactured using four different extrusion dies at Kansas State University (Manhattan, KS, USA). Ten samples of each of the 10 commercial kibble brands and the four custom-manufactured kibble sizes were analyzed for surface area, piece density, and bulk density. Bulk density was measured using a bulk density measuring device (Seedburo; Ohaus Scale Cooperation; Union, NJ, USA) and a scale (Mettler Toledo XP2003S; Columbus, OH, USA). Piece density was measured using a gas pycnometer (Micrometrics AccuPyc II 1340; Norcross, GA, USA). Surface area was measured by calipers, with 20 individual kibbles evaluated per sample. The surface area of cylindrical-shaped kibbles was calculated using the formula SA= $2\pi rh+2\pi r2$, with r being the radius and h being the height of the kibble. The surface area of triangular shaped kibbles was calculated using the formula SA = 3 (l×b) + [2(0.5b×h), with l being the length, b being the base, and h being the height of the kibble.

Preparation of Coated Samples. Samples were coated with sodium bisulfate (SBS; Jones-Hamilton, Co. Walbridge, OH) at differing levels for cat and dog foods (0, 0.6, and 0.8 vs. 0, 0.2, and 0.4%, respectively). Each type of kibble was coated with sodium bisulfate to match an industrial coating level of 0.25 kilograms per ton. Cat foods had a greater surface area than the dog food, and thus required a greater percentage of SBS. The SBS coating was mixed in a 1:1 blend of SBS and dry liver digest palatant (AFB International, C2737). For application, the kibble was mixed while the dry SBS-flavor blend was applied using a vibratory feeder over a period of one minute. The coated product was then mixed for an additional 3 minutes. The entirety of the coated kibble was then emptied into whirl-pak bags, and sub-samples were retained for *Salmonella* inoculation and analysis at the Kansas State University Grain and Feed Microbiology and Toxicology Laboratory (Manhattan, KS, USA). Other sub-samples were analyzed for sulfur (S) concentration at the Kansas State University Soil Testing Laboratory (Manhattan, KS, USA).

Preparation of Inoculum.

Experiment 1. Once coated kibble samples were obtained, a dry powder *Salmonella* cocktail inoculum was prepared. Briefly, samples of each kibble type were aseptically crushed into a powder, and 100 g sub-samples of the 0% concentration were directly inoculated with a 10 mL aliquot of the *Salmonella* enterica spp. Enteritidis culture cocktail (ATTC 13076). This provided an inoculum level of approximately 10^7 cfu/g of *Salmonella*. The inoculated powder was allowed to dry overnight in a biological safety cabinet. To confirm the concentration of the organism in the inoculated powder, an aliquot was enumerated. The inoculated powder was used

for all inoculations for coated foods during the 14 days of storage. Water activity and pH of the inoculated powder were compared to uninoculated powdered product of the same variety to ensure the inoculation method had not changed the properties of the inoculated, dried material.

Experiment 2. Each sample was inoculated with *Salmonella* enterica spp. Enteritidis ATCC#13076 using a wet inoculation technique. Briefly, one vial of bacteria per replicate was removed from the -80ºC freezer stock, and thawed. Next, 500 µL was inoculated into 10 mL tryptic soy broth (TSB; Difco; Franklin Lake, NJ, USA) tubes, one tube for each sample and coating level. These tubes were incubated at 35±2ºC for 48 hours. To prepare the culture for kibble inoculation, each tube was vortexed thoroughly. and 15 mL was transferred to a 15 mL Falcon tube (BD Biosciences, Bedford, MA). Once all cultures were transferred, they were centrifuged at 5,000 RPM for 5 minutes to pellet cells. After centrifugation, 7 mL was removed, and the remaining 3 mL of broth was used to re-suspend pelleted cells for inoculation.

Inoculation and Storage of Samples.

Experiment 1. Ten replicates were prepared for each of the 10 pet food products at each of the 6 SBS concentration (600 samples total). A 1 g portion of inoculated powder was mixed with each powdered sample to provide a target inoculation level of approximately 105 cfu *Salmonella*/g of product. An additional triplicate set of uninoculated, untreated subsamples were prepared as a background control. Inoculated subsamples were split into 5 groups of 4 samples; one group was tested immediately following inoculation, while the remaining groups were held at room temperature and tested after 1, 2, 7 and 14 days of storage.

Experiment 2. Three replicates were prepared for each of the 4 dog food kibble sizes at each of the 3 SBS concentration (36 samples total). Using sanitized hand sprayers, the concentrated *Salmonella* culture was sprayed onto kibble. After application, each kibble type was placed into a wide-mouth polypropylene, autoclavable container (Nalgene, Thermo Scientific, Waltham, MA USA) and were shaken vigorously and allowed to set for one hour before sampling for plate counts. Inoculated subsamples were split into 5 groups of 4 samples; one group was tested immediately following inoculation (day 0), while the remaining groups were held at room temperature and tested after 1, 2, 7 and 14 days of storage.

Sample Plating and Enumeration.

Experiment 1. The 25 g product samples were mixed with 225 mL of Butterfield's Phosphate Buffer (BPB) in a laboratory blender (Smasher, AES Chemunex, Durham, NC). The resulting homogenate was spread plated at appropriate dilutions onto Xylose Lysine Deoxycholate Agar (XLD, Neogen, Lansing, MI). XLD plates were incubated at 35 ± 2°C for 24 ± 2 hours.

After incubation, plates were enumerated using a Quebec colony counter. The number of observed colonies typical for *Salmonella* was multiplied by the dilution factor to determine the total count in cfu/g. Representative isolates were confirmed as *Salmonella* to ensure the recovered counts represented the inoculated culture. Counts for the untreated and uninoculated controls were also recorded.

The raw count observed for each sample was converted to $\log_{10}$ cfu/g. The quantity of *Salmonella* present in the treated samples (0.2% and 0.4% acidulant for dog foods; or 0.6% and

0.8% acidulant for cat foods) was compared to the quantity present in the untreated (0% acidulant) samples for each pet food variety to determine the total log reduction.

Experiment 2. To determine the initial concentration of *Salmonella* on the kibble, day 0 plate counts were performed. A total of 11 g of each of the samples was mixed with 99 mL of Buffered Peptone Water (BPW) in a whirl-pak bag and shaken vigorously. The resulting mixture was spread plated at appropriate dilutions onto Xylose Lysine Desoxycholate Agar (XLD, Neogen). XLD plates were incubated at 35 ± 2°C for 24 ± 2 hours. After incubation, plates were then enumerated. The number of observed colonies typical for *Salmonella* was multiplied by the dilution factor to determine the total count in cfu/g. Representative isolates were confirmed as *Salmonella* to ensure the recovered counts represented the inoculated culture. Counts for the untreated and uninoculated controls were also recorded. The raw count observed for each sample was converted to $\log_{10}$ cfu/g. The quantity of *Salmonella* present in the treated samples (0.2% and 0.4% SBS) was compared to the quantity present in the untreated (0% SBS) samples for each kibble variety to determine the total log reduction.

Palatability Testing (Exp. 3)

Diet. A single, uncoated, dry extruded basal diet manufactured for dogs of all life stages was obtained from a commercial pet food manufacturer prior to coating. The formula was proprietary; however, there were guarantees of 18% crude protein and 6% crude fat.

Kibble Coating. The kibble was coated with either 2.2% spray dried chicken liver (Kemin; Des Moines, IA USA) + 0.2% sodium bisulfate (SBS; Jones-Hamilton, Co., Walbridge, OH) or 2.2%

spray dried chicken liver + 0.2% powdered silica (control). The mixture of SBS and spray dried chicken liver, or powdered silica and spray dried chicken liver was mixed prior to application to the kibble. For application, a total of 22.68 kg of uncoated kibble for each treatment was added to an empty cement mixer. The kibble was mixed while the dry flavor blend was applied using a vibratory feeder over a period of eight minutes. The coated product was then mixed for an additional 5 minutes. The entirety of the coated kibble was emptied into paper feed sacks, and sealed using a bag seamer. Each treatment was transported to a commercial kennel facility for palatability testing (Kennelwood, Inc., Champaign, IL USA).

Palatability Testing. A total of 20 beagles were used in a standard two-bowl forced choice palatability test method for two days. Dogs were fed 400 g of both diets once per day, with bowls rotated daily to address side bias.

Statistical Analysis.

Exp. 1 and 2. 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 SBS inclusion level, kibble size, and their interactions, with sampling day serving as a repeated measure. There were three replicates of each kibble size and SBS inclusion level. Differences were considered statistically significant at $P < 0.05$.

Exp. 3. Results were analyzed using the GLIMMIX and FREQ procedures of SAS version 9.3 (Cary, NC). In the GLIMMIX procedure, there were fixed effects of SBS inclusion, and dog food consumption, with sampling day serving as a repeated measure. The FREQ

procedure was performed to establish the significance in first choice preference. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

In Exp. 1 and 2, SBS inclusion level significantly reduced post-processing *Salmonella* contamination in dog and cat foods ($P < 0.001$; Table 3.1). It was hypothesized that sodium bisulfate would need to be included at higher levels in kibble with more surface area per volume, but surprisingly, surface area did not influence ($P > 0.05$) the effectiveness of SBS on reducing *Salmonella* concentration over time. As surface area increased, the concentration of SBS did not appear to increase ($P > 0.05$) in effectiveness against *Salmonella* concentration; this may be related to other factors between the two types of food, such as kibble size (significantly larger for the dog food), shape, or other ingredients. In Exp. 1, there were no significant interactions between surface area, bulk density, or piece density and the effect of *Salmonella* mitigation ($P > 0.05$). However, the piece density and the *Salmonella* concentration were highly correlated (Table 3.1). In Exp. 1, cat foods coated with the lowest quantity of SBS, 0.6%, had *Salmonella* concentrations reduced ($P < 0.05$) by 3.24 log compared to uncoated cat foods by d 14. The quantity of *Salmonella* in dog food coated with the lowest tested level of SBS, 0.2% was reduced ($P < 0.05$) by an average of 2.85 log by d 14. However, there were no significant differences between the low and high coating level within species ($P > 0.05$). *Salmonella* concentration decreased linearly with time ($P < 0.05$; Table 3.2).

In Exp. 2, there was no effect of kibble size, or its interactions, on *Salmonella* concentration ($P > 0.10$; Table 3.3). This is in contrast to a study performed by Xue et al., (2011), surface area was an important factor in the effectiveness of a reaction involving sodium bisulfate. We did not see these differences potentially because of the particle size of the meal

prior to extrusion was relatively similar, regardless of the different sizes of kibbles. There was a significant effect of time, coating level, and their interaction on *Salmonella* reduction ($P < 0.001$). There were no effects between surface area, bulk density or piece density and the effect of *Salmonella* mitigation.

In Exp. 2, levels of *Salmonella* in dog food coated at 0.2% SBS were reduced ($P < 0.05$) from an average of 1.6 logs over the course of the 14 days. Levels of *Salmonella* in dog food coated at the highest level (0.4% SBS) were reduced ($P < 0.05$) from an average of 2.0 logs over the course of the 14 days. There were no significant differences between the 0.2% and the 0.4% coating levels ($P > 0.05$; Table 3.4).

These results agree with a study performed by Pope and Cherry (2000), who showed that the use of SBS as a coating exhibits considerable antibacterial activity, resulting in a 2 to 3 log reduction of bacterial populations. SBS has a two-fold mechanism of action; first, it reduces the level of pH in a primary matrix (Kassem et al., 2012). *Salmonella* growth exists during two ranges of pH: free growth at a pH of approximately 7, and as an intracellular pathogen that will grow into macrophages at a pH range of approximately 4.5 to 5. When the SBS initially lowers the pH of the primary matrix, the *Salmonella* cells are subjected to an unfavorable environment, thus free growth stops. The second mode of action of SBS is through hygroscopcity, or dessicant action (Sun et al., 2008). Thus, SBS draws fluid out of the *Salmonella* cell cytoplasms, causing apoptosis of the cells.

Time, coating level, and their interactions all influenced ($P < 0.05$) *Salmonella* concentrations of pet foods coated with SBS, while kibble size had no effect ($P > 0.05$). This suggests that regardless of the type of pet food, sodium bisulfate is an effective method of reducing post-processing *Salmonella* contamination. There are other methods of *Salmonella*

mitigation, including the use of bacteriophage cocktails (Heyse et al., 2015). In that study, a cocktail made of six different bacteriophages had a broad spectrum antibacterial activity against 930 *Salmonella* enterica strains, representing over 44 known serovars. The bacteriophage cocktail was effective against 95% of the tested strains. This appears to be effective, however, in the feed industry, it presents hazards of cross-contamination, which is what we are ultimately trying to prevent. The bacteriophage cocktail may contaminate other feeds, and also may be hazardous to the workers in the production facility.

Palatability Experiment (Exp. 3)

While SBS represents a better mitigation technique against *Salmonella* in pet food, its industrial use within the pet food industry depends on the palatability of SBS, which has not been evaluated. There were no significant differences of consumption when comparing the control diet vs. the SBS diet on both days separately ($P > 0.05$). Thus, the inclusion of SBS did not affect daily preference of diet ($P > 0.05$, Table 3.6). There was also no effect of day ($P > 0.05$), or the interaction of treatment × day ($P > 0.05$). There were no significant differences in the first approach and first choice preferences ($P > 0.05$).

The use of SBS may be a more promising method of *Salmonella* mitigation, especially due to there being no discernible effects on palatability of the dog food studied. However, only beagles were used in this particular study. Beagles have been shown to have less discrimination of one diet compared to another, when compared to other breeds of dogs (Ferrell, 1984). Beagles may or may not have the same taste preferences as other breeds of dogs. Thus, further research considering the palatability of SBS as a coating in other dog foods, and other breeds of dogs is warranted. Also, research on the palatability of SBS as a coating in cat food needs to be

performed before it is safe to say that SBS is palatable in pet foods. However, since most cat food is already acidic (Pickering, 2007), there will likely be no effect on the palatability of SBS on cat food. Overall, coating dog and cat foods with SBS is very effective at reducing the amount of *Salmonella* over time at the lowest tested concentration.

Overall Conclusions

Our research confirmed that sodium bisulfate is an effective mitigation technique for reducing surface *Salmonella* concentrations in pet foods over a 14 day storage time period at coating levels of 0.2% and 0.4% in dog food, and 0.6% and 0.8% in cat food. In some dog food, the 0.4% provided complete *Salmonella* mitigation by day 2, and the 0.4% coating level provided complete mitigation by day 7. Sodium bisulfate acts as an acidulant to lower the pH, in addition to acting as a dessicant. This two-fold mechanism allows for apoptosis of *Salmonella* already present via drawing water out of the cytoplasm(s), and prevents growth via reducing the pH, making for an unfavorable environment for *Salmonella* growth. Sodium bisulfate is used in industrial and agricultural applications, and has been used for years as an acidifier to reduce pH in pet diets and is classified as a general purpose feed additive by the Association of American Feed Control Officials (AAFCO; Ruiz-Feria et al., 2011).

Since 0.2% and 0.4% coating levels were effective at reducing *Salmonella* concentrations in Experiment 1 and 2, it is possible that a lower level of inclusion may be effective, but further research for lower inclusion levels is warranted. Overall, SBS appears to be a promising method of *Salmonella* mitigation in the pet food industry.

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Tables

Table 3.1 Exp. 1. Effects of sodium bisulfate (SBS) coating concentration in commercially-produced cat or dog food on *Salmonella* concentration over time[1]

	SBS Concentration[2]		
Item;	None	Low	High
Cat food *Salmonella*, $\log_{10}$ cfu/g			
d 0	4.43[a]	4.41[a]	4.29[a]
d 1	3.73[b]	3.22[cd]	3.16[cd]
d 2	3.01[de]	2.56[f]	2.63[ef]
d 7	3.21[cd]	1.88[g]	1.38[h]
d 14	3.47[bc]	1.31[h]	1.01[h]
Dog food *Salmonella*, $\log_{10}$ cfu/g			
d 0	4.84[a]	4.93[a]	4.95[a]
d 1	3.56[bc]	3.64[b]	3.61[bc]
d 2	3.64[bc]	2.67[e]	2.51[e]
d 7	3.20[d]	2.22[f]	2.05[fg]
d 14	3.36[cd]	2.09[f]	1.92[g]

Factor	*P* =	SEM
Pet food type	0.038	0.069
SBS concentration	< 0.0001	0.063
Time[3]	< 0.0001	0.057
Pet food type × SBS concentration	< 0.0001	0.099

[1]Ten commercially extruded pet foods (5 dog and 5 cat foods) were treated with SBS prior to inoculation with *Salmonella*, and analyzed for *Salmonella* concentration on d 0, 1, 2, 7, and 14.

[2]Coating levels of SBS for cat foods were 0, 0.6, and 0.8% and for dog foods were 0, 0.2, and 0.4% for the none, low, and high SBS concentrations, respectively.

[3]Linear effect, $P < 0.05$.

[abcdefgh]Values within a row and column in a single food type that do not share a common superscript differ $P < 0.05$.

Table 3.2. Exp. 1. Effects of cat or dog food type on resultant *Salmonella* concentration and product characteristics[1]

	Product Type			
Item;	Cat	Dog	SEM	*P* =
Salmonella, $\log_{10}$ cfu/g	2.91	3.28	0.125	0.04
Sulfur, %	0.61	0.36	0.026	< 0.0001
Bulk Density, g/L	473	452	25.2	0.56
Piece Density, g/cm^3	1.33	1.37	0.014	0.08
Surface area, mm^2	628	917	257.0	0.45

[1]Ten commercially extruded pet foods (5 dog and 5 cat foods) were treated with SBS prior to inoculation with *Salmonella*, and analyzed for *Salmonella* concentration on d 0, 1, 2, 7, and 14.

Table 3.3. Exp. 2. Effects of sodium bisulfate (SBS) coating concentration in dry extruded dog food on *Salmonella* concentration over time[1]

	SBS Concentration[2]		
Item;	None	Low	High
Dog food *Salmonella*, $\log_{10}$ cfu/g			
d 0	4.47[a]	4.06[ab]	3.76[bc]
d 1	3.85[bc]	2.88[d]	2.39[e]
d 2	3.46[c]	0.95[g]	Undetected[h]
d 7	2.68[de]	0.22[h]	Undetected[h]
d 14	1.91[f]	0.15[h]	Undetected[h]
Factor	*P* =	SEM	
SBS concentration	<0.0001	0.097	
Time[3]	< 0.0001	0.126	
Time × SBS concentration	< 0.0001	0.219	

[1]Four different kibble sizes of a single dry extruded dog food were treated with a dry acidulant, sodium bisulfate (SBS) at coating levels of 0.0%, 0.2%, and 0.4%, and were analyzed for *Salmonella* concentration on d 0, 1, 2, 7, and 14.

[2]Coating levels of SBS for dog foods were 0, 0.2, and 0.4% for the none, low, and high SBS concentrations, respectively.

[3]Linear effect, $P < 0.05$.

[abcdefgh]Values within a row and column in a single food type that do not share a common superscript differ $P < 0.05$.

Table 3.4. Exp. 2. Effects of dog food type on resultant *Salmonella* concentration and product characteristics[1]

	Product Type		
Item;	Dog	SEM	*P* =
Salmonella, $\log_{10}$ cfu/g	3.272	3.2715	<0.0001
Bulk Density, g/L	356.2	80.79	0.0217
Piece Density, g/cm^3	0.786	0.0616	0.001
Surface area, mm^2	2453	1633.29	0.2302

[1]Four different kibble sizes of a single extruded dog food were treated with a dry acidulant, sodium bisulfate (SBS) at coating levels of 0.0%, 0.2%, and 0.4%, and were analyzed for *Salmonella* concentration on d 0, 1, 2, 7, and 14.

Table 3.5. Exp. 2. Correlation among *Salmonella* concentration, bulk density, piece density, and surface area[1]

	Correlation with *Salmonella*	
Variable;	Coefficient	*P* =
Bulk Density	0.40	0.1216
Piece Density	0.23	0.3919
Surface Area	0.23	0.3888

[1]Four different kibble sizes of a single extruded dog food were treated with a dry acidulant, sodium bisulfate (SBS) at coating levels of 0.0%, 0.2%, and 0.4%, and were analyzed for *Salmonella* concentration on d 0, 1, 2, 7, and 14.

Table 3.6. Palatability of Dog Food Coated with Sodium Bisulfate

	Daily Feed Disappearance (g)		
Item	0.2% silica control	0.2% SBS	*P* =
Day 1	116.25[a]	98.85[a]	0.98
Day 2	93.25[a]	73.5[a]	

[a]Values within a row and column that do not share a common superscript differ $P < 0.05$.
[1]20 dogs were utilized in a 2 day, two-bowl palatability test method.

Table 3.7. Effect of sodium bisulfate addition on first approach in dogs

	First Approach (n=)		
Item	0.2% silica control	0.2% SBS	*P* =
Day 1	11[a]	9[a]	0.20
Day 2	13[a]	7[a]	

[a]Values within a row and column that do not share a common superscript differ $P < 0.05$.

[1]20 dogs were utilized in a 2 day, two-bowl palatability test method.

Table 3.8. Effect of sodium bisulfate addition on first choice in dogs

	First Choice (n=)		
Item	0.2% silica control	0.2% SBS	*P* =
Day 1	9[a]	11[a]	0.53
Day 2	9[a]	11[a]	

[a]Values within a row and column that do not share a common superscript differ $P < 0.05$.
[1]20 dogs were utilized in a 2 day, two-bowl palatability test method.

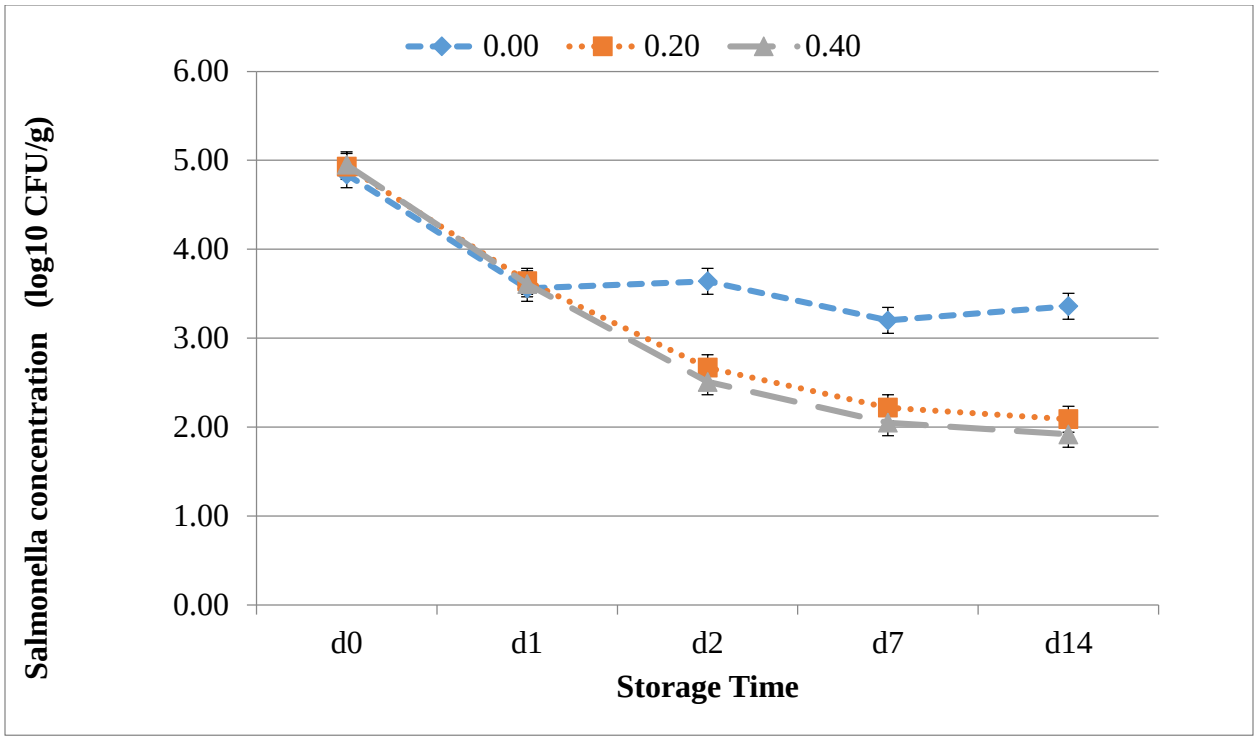

Figure 3.1. Effect of sodium bisulfate (SBS) coating on *Salmonella* contamination in commercial dog food (Experiment 1).

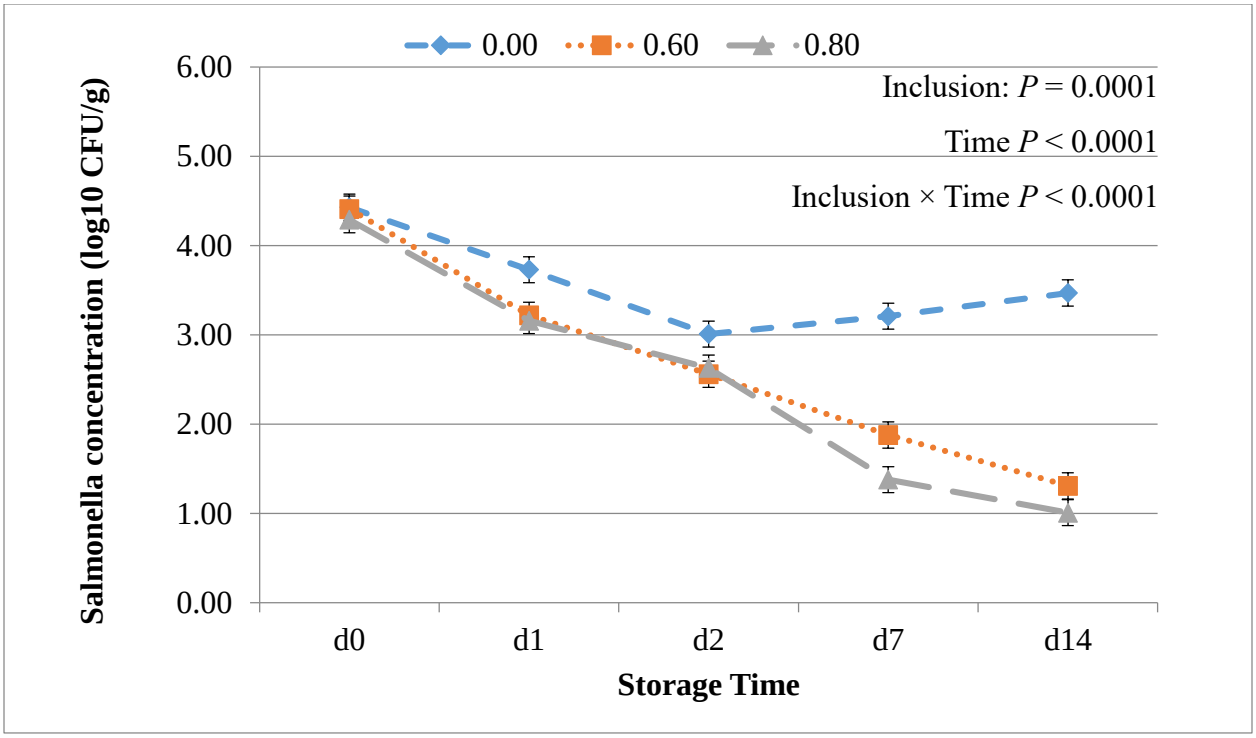

Figure 3.2. Effect of sodium bisulfate inclusion level on *Salmonella* concentration in commercial cat food (Experiment 1).

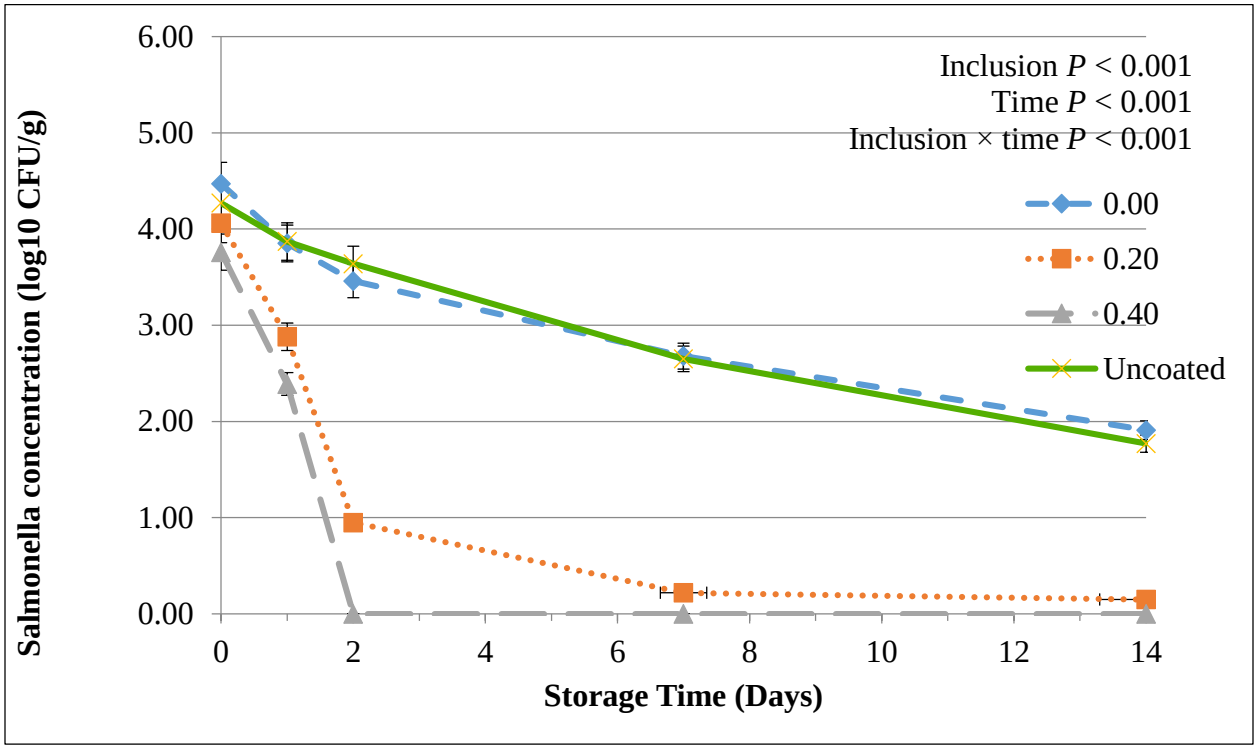

Figure 3.3. Effects of sodium bisulfate inclusion level on salmonella concentration in dog food prior to enrobing (Experiment 2).

Chapter 4 - Effects of a dry acidulant addition to prevent *Salmonella* Contamination in Poultry Feed

Summary

Salmonella subs. serovar enteritidis is a potential biological pathogen of concern in the poultry industry. Contamination of the bacterium on shell eggs has led to human illnesses. With the implementation of new regulations, animal feed manufacturing continues to be under more stringent requirements. Specifically, there is zero tolerance for *Salmonella* Pullorum, Gallinarum, or Enteritidis in poultry feed. For this reason, it is important to determine an effective method of reducing or preventing *Salmonella* contamination in feed for poultry. Therefore, the objective of this study was to evaluate the impact of sodium bisulfate (SBS; Jones-Hamilton, Co., Walbridge, OH) added to poultry mash to reduce or prevent *Salmonella* growth over time. A single, commercially-produced all-flock poultry mash was mixed with four different levels of SBS: 0.0, 0.25, 0.50, and 0.70%. After SBS addition, the treated mash was inoculated with *Salmonella enterica* subsp, *enterica* Serovar enteritidis (ATCC 13076) and enumerated for *Salmonella* on day 0, 1, 2, 7, and 14 post-inoculation by plating to XLD agar. There was no significant effect of SBS inclusion level on the reduction of *Salmonella* ($P = 0.23$); however, there was a significant effect of time across treatments ($P < 0.0001$). Additionally, there was no impact of the interaction of inclusion level × time ($P = 0.68$). Keeping these results and other research in mind, it is important to continue to search for more effective methods to reduce or prevent *Salmonella* contamination in poultry feed.

Introduction

Non-typhoidal *Salmonella* are estimated to globally cause 93.8 million infections and 155,000 deaths each year (Varga et al., 2013). Approximately 11% of *Salmonella* infections are attributed to animal exposure annually (Mead et al., 1999; Hale et al., 2012). Thus, animal feed is at the beginning of the food safety chain in the farm-to-fork model. Animal food may be contaminated through cross-contamination during manufacturing at the feed mill (Crump et al., 2002). Contaminated animal feed may, in turn, lead to infection of food producing animals, including poultry. While infrequently linked, pathogens including *Salmonella*, may then be transmitted through the food chain to humans, causing human foodborne illness.

Salmonella is a gram negative bacteria that can be found in the intestinal tracts of many animals, including poultry (Heymann, 2008; Behravesh et al., 2014). Poultry are well recognized as carriers of *Salmonella*, and may appear healthy while infected (Behravesh et al., 2014), thus posing the risk for zoonotic disease transfer to humans from birds that appear healthy. Some strains of non-typhoidal *Salmonella* have greater survivability in animal feed than others (Andino, 2014). Reducing *Salmonella* contamination in animal feed may result in fewer *Salmonella* infections of flocks thus reducing the risk of contamination to the human food supply.

There are several options to control *Salmonella* contamination in animal food. The time and temperature combination reached during the extrusion process should result in *Salmonella*-free kibble production, but does not prevent post-processing contamination (Baldwin and Rokey, 2013). Chemical addition, such as commercial formaldehyde, medium chain fatty acids, organic acids, or essential oils to ingredients and finished swine feeds have been shown to effectively reduce *Salmonella* contamination (Cochrane et al., 2015).

It has been well established that acidifiers can reduce pH and prevent *Salmonella* growth and viability (Humphrey, 1988; Matlho et al., 1997; and Koyuncu et al., 2013). These additives are also an attractive option to utilize in feed safety because many are currently included in poultry diets to improve the digestibility of amino acids (Kassem et al., 2012). However, use of these chemicals have disadvantages because many are hazardous for employees to handle, they may cause corrosion of manufacturing equipment, and they are traditionally liquid, which requires specialized application equipment for accurate inclusion.

An alternative acidifier that is generally recognized as safe (GRAS) by the FDA is sodium bisulfate (SBS; Jones-Hamilton Co., Walbridge, OH USA). The additive is a weak acid in a bulk, dry, powdered form and currently approved for inclusion in poultry feed to reduce pH. Theoretically, the acidification action of SBS may reduce the pH of poultry feed to prevent bacteria growth. Additionally, the product has a desiccant action that has the potential to draw moisture from within *Salmonella* cells, reducing the viability of the bacterium. The acidulant has been demonstrated to substantially reduce *Salmonella* when applied as a coating in pet foods (Jeffrey et al., 2014). Inclusion of SBS into poultry feed is an attractive alternative to other chemical additives due to its current use in the poultry industry with fewer negative implications on palatability, worker safety, or equipment surfaces as other liquid acidulants (European Food Safety Authority, 2014). Therefore, the objective of this study was to evaluate the ability of SBS added to poultry mash to reduce or prevent *Salmonella* growth over time.

Materials and Methods

***SBS Inclusion*.** A single, commercially-produced all-flock poultry mash (Country Lane, Moberly, MO) was coated with the powdered form of SBS. The untreated nutritional analysis

and ingredient list of the poultry mash is given in Table 4.1. SBS was added at the final inclusion levels of 0.0, 0.25, 0.50, and 0.70% w:w to mimic industrial coating levels typically used in poultry mash formulations. For each level of inclusion, SBS was mixed thoroughly for an even distribution.

Salmonella Inoculum Preparation and Inoculation. *Salmonella enterica* subsp, *enterica* Serovar Enteritidis (ATCC 13076) stock stored at -80°C was transferred to fresh tryptic soy broth (TSB; Difco, Franklin Lakes, NJ USA) and incubated at 35º for 48 hours. Following incubation, the cultured TSB was added to 100 g of previously sterilized, uncoated poultry mash and allowed to dry overnight in the biosafety cabinet. The prepared inoculum was then mixed with the previously treated poultry mash at each of the four inclusion levels. Treated and inoculated poultry mash was shaken vigorously for 2 minutes to evenly distribute the inoculum. Following inoculation the poultry mash was allowed to sit at room temperature for one hour prior to the collection of day 0 enumeration. Additionally, all inoculated treatments were stored at room temperature throughout the 14 day sampling period.

Sample Plating and Enumeration. Enumeration of *Salmonella* was carried out on days 0, 1, 2, 7, and 14 post-inoculation. For each day of enumeration, subsamples were collected, diluted with Buffered Peptone Water (BPW; Difco, Franklin Lake, NJ) and stomached for 30 s in a stomacher (Seward 400, Davie, FL). Following sample stomaching, serial dilutions in BPW were performed and spread plated onto Xylose Lysine Deoxycholate agar (XLD; Difco, Franklin Lake, NJ). All inoculated XLD plates were incubated at 35 ± 2°C for 24 ± 2 hours. After incubation, black

colonies typical for *Salmonella* were counted and total colony forming units (CFU) per g calculated.

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 SBS inclusion level, with sampling day serving as a repeated measure. There were three replicates of each SBS inclusion level. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

The level of SBS did not impact *Salmonella* concentration ($P = 0.23$; Figure 4.1). These results agree with various studies performed to determine the effect of sodium bisulfate on mitigation of *Salmonella enteritidis* serovars (Williams, et al., 2012; Line and Bailey, 2006). Line and Bailey (2012) studied the effect of sodium bisulfate on *Salmonella* counts in broiler houses. The results demonstrated that the inclusion of sodium bisulfate had no significant effect on the *Salmonella* counts in the litter samples. Williams et al. (2012) also performed a similar study to evaluate the effect of sodium bisulfate on *Salmonella* in poultry litter. In this study, sodium bisulfate was included at varying rates of application, and samples were analyzed over a 6 week period. There was no significant effect of the inclusion of sodium bisulfate, which agrees with our results, and the results from the Line and Bailey (2012) study.

Acidifiers, including sodium bisulfate, are currently being used in the poultry industry as agents to reduce pH in poultry feeds (Kassem et al., 2012), and may be used as an antimicrobial agent in poultry feeds to reduce or eliminate *Salmonella* contamination within the industry. In particular, the acidulant sodium bisulfate has been used as an agent to reduce pH in several

industrial and agricultural applications and as an acidifier in pet diets, and has been classified as a general purpose food additive under the Association of American Feed Control Officials Feed Ingredient Definition (AAFCO; Jeffrey et al., 2014; Ruiz-Feria, 2011). It is also used as a supplement to treat farm animal litter, where considerable antibacterial is observed, resulting in 2 to 3 logs reduction in the litter's bacterial populations (Pope and Cherry, 2000). The main mode of action of sodium bisulfate is its hygroscopicity (Sun et al., 2008). As moisture is absorbed into the SBS, the compound dissolves into a sodium ion (Na^+), hydrogen (H^+), and a sulfate ion (SO_4^-) (Sun et al., 2008; Jones-Hamilton, Walbridge, OH). It is possible that the sodium bisulfate was not included at high enough levels to coat all of the feed particles evenly, in order to have a significant effect on *Salmonella* concentration. There have been other findings showing the significant effect on *Salmonella* concentration over time in other feed ingredients and pet food (Cochrane et al., 2016; Jeffrey et al., 2015); however, the pet food was in a kibble matrix. The mash form of the diet in this experiment may have also contributed to the growth of *Salmonella,* because the nutrients are more readily available than in a kibble matrix. According to the European Food Safety Authority, sodium bisulfate as an additive is safe for consumers, users and the environment when used at the proposed maximum content of 1% in complete feedingstuffs (EFSA, 2012).

While SBS did not impact *Salmonella* concentration, time substantially reduced ($P < 0.0001$) the bacteria, regardless of the inclusion level of SBS (Figures 4.2 and 4.3). At the end of the 14 day storage period, the level of *Salmonella* in all treatments was undetectable. These results are also demonstrated in a different study (Williams et al., 2012) evaluating the effects of SBS in poultry litter. While the same matrix is not used, the same general trend was seen, where *Salmonella* decreases over time; however, the level of SBS inclusion does not have

any significant effect (Williams et al., 2012). As feed is stored, water activity decreases, which is presumably the reason for the impact of time on *Salmonella* concentration. This in itself is a significant finding, as feed storage may be an effective step to reducing bacterial contamination. A storage time period of 14 days minimizes the opportunity for bacterial pathogen contamination. However, this may not be practical because most poultry feed in integrated systems is fed within hours or days of manufacturing.

In summary, time, but not dry acidulant addition, impacted *Salmonella* Enteritidis concentration in mash poultry diets. A 14-d storage time sufficiently eliminates the bacteria to indictable levels, but is likely impractical for most poultry producers. Thus, further research is needed to identify effective and practical methods to reduce *Salmonella* Enteritidis contamination in poultry feed.

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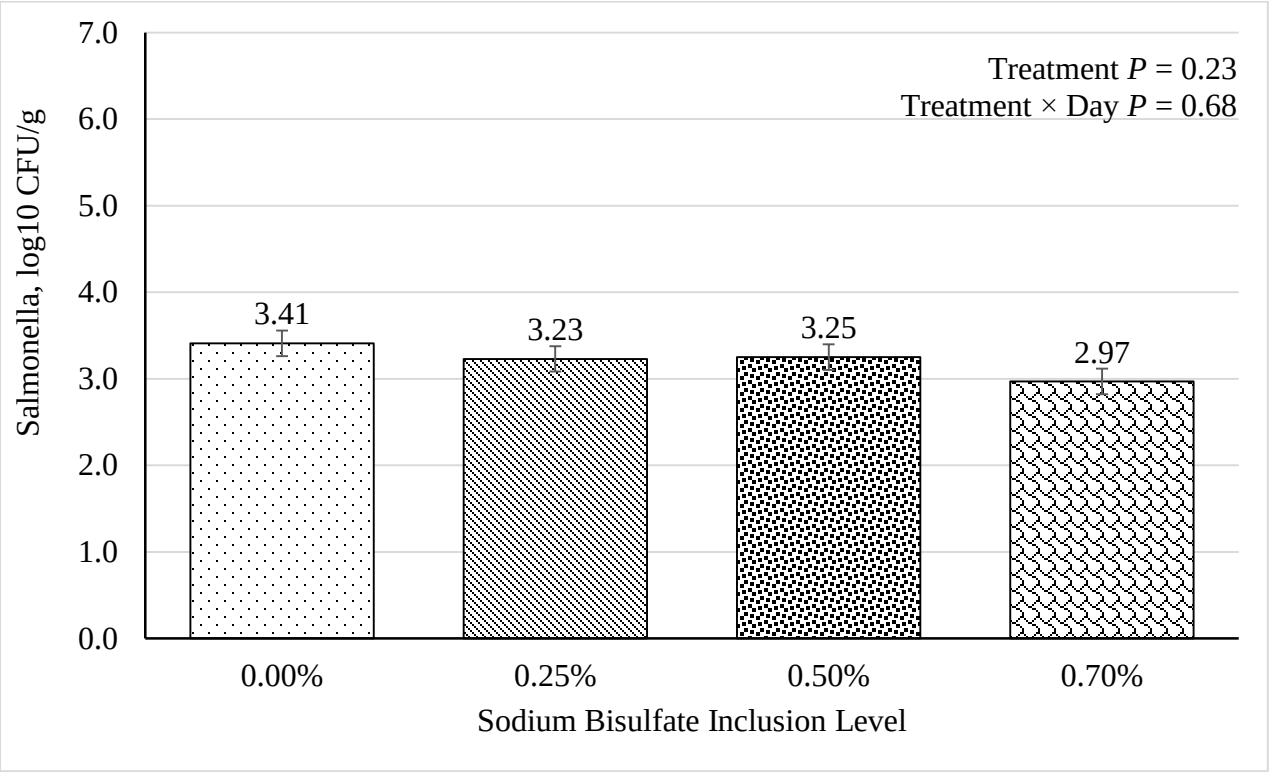
Figures and Tables

Table 4.1. Formulation and ingredient list[1] of the all-flock mash

Ingredient	Guaranteed Analysis, %
Crude Protein (min)	16.00
Lysine (min)	0.60
Methionine (min)	0.30
Crude Fat (min)	3.00
Crude Fiber (max)	9.00
Calcium (min)	1.50
Calcium (max)	2.00
Phosphorous (min)	0.50
Salt (min)	0.25
Salt (max)	0.75
Sodium (min)	0.15
Sodium (max)	0.65
Vitamin A (min)	3,000 IU/lb
Vitamin E (min)	20 IU/lb

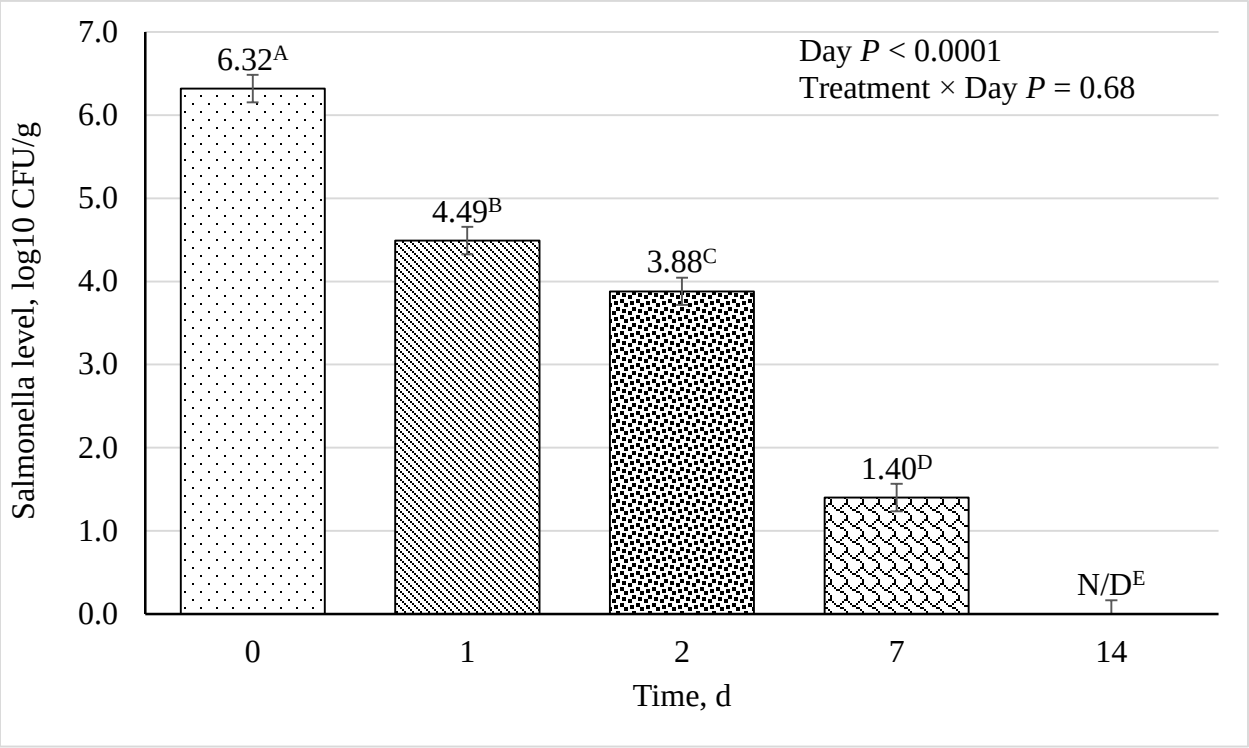
[1]Processed grain by-products, grain products, plant protein products, calcium carbonate, sodium bentonite, salt, l-lysine, dl-methionine, ferrous carbonate, ferrous sulfate, copper sulfate, manganous oxide, manganese sulfate, zine oxide, zinc sulfate, cobalt carbonate, sodium selenite, vitamin A supplement, vitamin D3 supplement, vitamin E supplement, menadione dimethylprimidinol bisulfite, thiamine mononitrate, riboflavin supplement, niacin supplement, choline chloride, calcium pantothenate, pyroxidine hydrochloride, folic acid, biotin, vitamin B12 supplement, propionic acid (a perservative)

Figure 4.1. Effect of dry acidulant inclusion level on *Salmonella* Enteritidis level[1, 2]



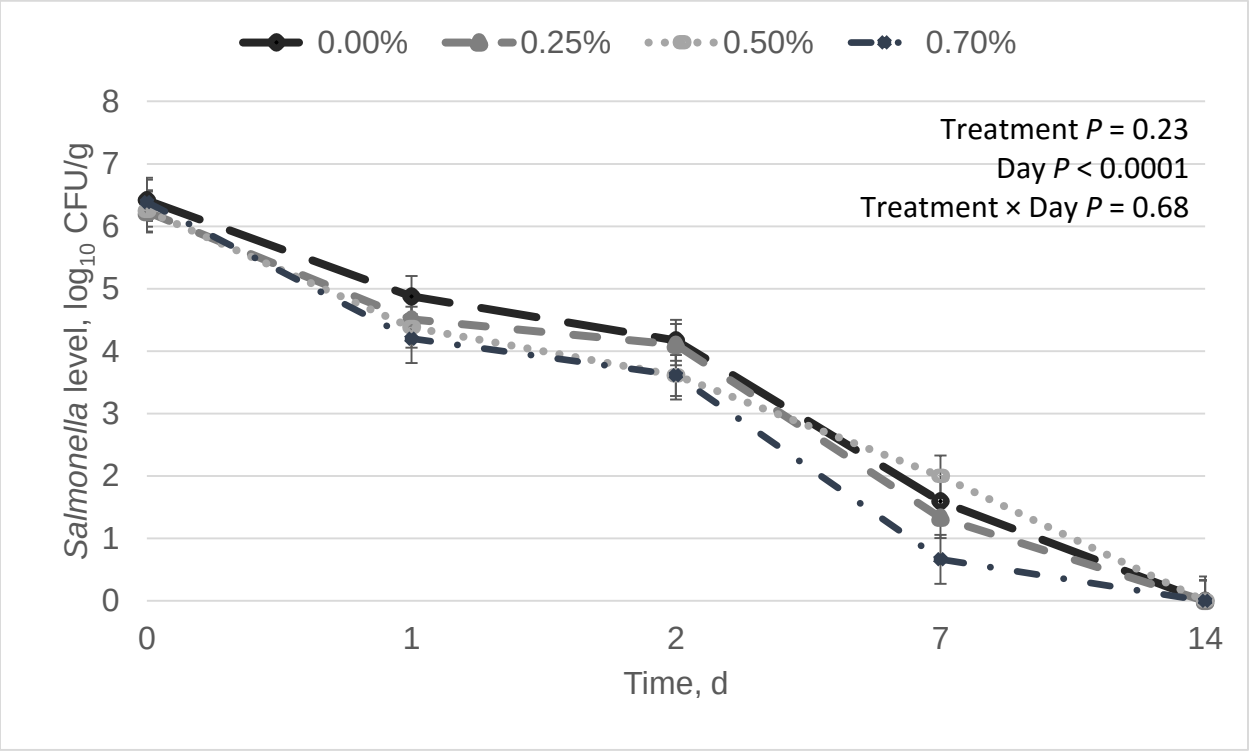
[1]A single, commercially produced all-flock poultry mash was treated with a dry acidulant, sodium bisulfate, at 0.0%, 0.25%, 0.50%, and 0.70%. On d 0, samples were inoculated with *Salmonella* and enumerated for *Salmonella* on Xylose Lysine Deoxycholate agar. Stored samples were enumerated for *Salmonella* Enteritidis days 1, 2, 7, and 14 post-inoculation.
[2] Means lacking a common superscript differ $P < 0.05$

Figure 4.2. Effect of day on *Salmonella* Enteritidis level[1, 2]



[1]A single, commercially produced all-flock poultry mash was treated with a dry acidulant, sodium bisulfate, at 0.0%, 0.25%, 0.50%, and 0.70%. On d 0, samples were inoculated with *Salmonella* and enumerated for *Salmonella* on Xylose Lysine Deoxycholate agar. Stored samples were enumerated for *Salmonella* Enteritidis days 1, 2, 7, and 14 post-inoculation.
[2] Means lacking a common superscript differ $P < 0.05$

Figure 4.3. Effects of dry acidulant inclusion level on *Salmonella* Enteritidis over time[1]



[1]A single, commercially produced all-flock poultry mash was treated with a dry acidulant, sodium bisulfate, at 0.0%, 0.25%, 0.50%, and 0.70%. On d 0, samples were inoculated with *Salmonella* and enumerated for *Salmonella* on Xylose Lysine Deoxycholate agar. Stored samples were enumerated for *Salmonella* Enteritidis days 1, 2, 7, and 14 post-inoculation.
[2] Means lacking a common superscript differ $P < 0.05$