EVALUATION OF DIFFERENTIAL SCANNING CALORIMETRY AS A POTENTIAL METHODOLOGY TO ASSESS STARCH GELATINIZATION IN COMMERCIAL POULTRY DIETS THERMALLY PROCESSED UNDER MULTIPLE RETENTION TIME AND TEMPEREATURE COMBINATIONS

by

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Abstract

Starch is known to gelatinize in the presence of heat and water during thermal processing; thus, causing the unraveling of the starch granule. The occurrence of starch gelatinization in diets having high inclusions of carbohydrates during thermal processing has been suggested to improve energy digestibility in production animals such as swine and poultry. Experiments were conducted to assess starch gelatinization (**SG**) and total starch (**TS**) content in multi-component animal food using Differential Scanning Calorimetry (**DSC**) and Association of Official Analytical Chemists (**AOAC**) 996.11 methodologies, respectively. DSC was utilized to assess SG in cooked and uncooked corn starch (**CS**), cooked and uncooked ground corn, uncooked meal, and pelleted animal food when set to a conclusion temperature of either 160 or 200 °C. Enthalpy (H_1) differed among cooked starch sources ($P = 0.0080$) and DSC temperature ($P = 0.0429$). No significant interaction was observed between starch source and DSC temperature ($P = 0.2166$) for cooked samples. Calculated DG of CS was 71.3 %, while the DG for GC and pelleted animal food could not be calculated because the uncooked enthalpy (H_0) for both starch sources was greater than the cooked enthalpy (H_1).

Nine treatment combinations were analyzed with 3 conditioning temperatures (**CT**; 75, 85, and 95 °C) and 3 retention times (**RT**) in the Hygieniser® (80, 160, and 240 s); samples (n = 198) for DSC analysis of SG were collected on 3 separate d. Data for all experiments were analyzed using the GLIMMIX procedure of SAS (V 9.4) and all means were separated utilizing the PDIFF option of SAS. Means were declared significantly different at $P \leq 0.05$.

Post- conditioner samples conditioned at 75 ℃ had higher enthalpy ($P = 0.0182$) than samples conditioned at 85 and 95 ℃. Enthalpy of samples collected after the Hygieniser® and conditioned at 75 °C were higher ($P = 0.0486$) than samples conditioned at 85 °C. An interaction ($P = 0.0200$) was observed for the enthalpy of samples collected after pelleting and conditioned at 75 ℃ and retained in the Hygieniser® for 80 s having the greatest enthalpy (54.9 J/g) than all other CT and RT combinations. The combination of proteins, fats, and other carbohydrate fractions do not appear to allow for the use of DSC. Based on these results, DSC is not a suitable method to assess SG in multi-component animal food.

AOAC 996.11 was utilized to assess TS in multi-component animal food using 9 treatment combinations with 3 CT and 3 RT. Conditioning temperature had the greatest effect on TS content of samples collected after the conditioner at 95 °C compared to 75 and 85 °C ($P = 0.0002$). Samples collected after the Hygieniser® and conditioned at 75 °C had the highest amount of TS compared to 85 and 95 °C ($P = 0.0001$). The pelleted samples had the highest percentage of TS ($P = 0.0152$) at 160 s, but the samples obtained after the Hygieniser® had the highest TS percentage when retained for 80 s ($P = 0.9339$). A significant interaction was observed between CT and RT in relation to TS content (%) for the after pelleted samples ($P \leq 0.0034$). No differences were observed between CT and RT for the TS content of the after Hygieniser® samples ($P = 0.853$). The highest percentage of TS occurred in the Hygieniser® at 85 °C for 240 s. AOAC 996.11 is only utilized to assess the percentage of TS present in a sample. To further determine the amount of SG that occurred in this diet using 9 treatment combination with 3 CT and 3 RT, the glucoamylase method must be utilized.

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List of Abbreviations

AC	After Conditioning
AH	After Hygieniser®
AOAC	Association of Official Analytical Chemists
AP	After Pelleting
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Units
CT	Conditioning Temperature
FDA	Food and Drug Administration
GMPs	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Point
RT	Retention Time
SE	*Salmonella* Enteritidis
SG	Starch Gelatinization
ST	*Salmonella* Typhimurium
TS	Total Starch

CHAPTER I: LITERATURE REVIEW

INTRODUCTION

Salmonella is a ubiquitous organism that is isolated from the gastrointestinal tract of food-producing animals, shed feces, contaminated raw ingredients, as well as from the farm, the environment, and animal food manufacturing facilities. *Salmonella* can be isolated from animal food, but the strains that are most commonly isolated are those that are non- pathogenic. Animal food can become contaminated through dust, animal carriers, or from personnel not following proper Good Manufacturing Practices (**GMPs**), and aseptic technique procedures when sampling food (Huss, 2015). Various methods have been implemented in food manufacturing facilities to assist in the mitigation of *Salmonella*, such as the use of chemicals or thermal inactivation. One example of thermal inactivation techniques that are utilized in industry is conditioning and pelleting. These thermal processing methods have shown a reduction in the prevalence of *Salmonella* in pelleted food compared to unprocessed meal due to pelleted food being processed under specific time and temperature parameters (Liu et al.,1969). Thermal processing can eliminate the majority of microorganisms, but pathogenic organisms may be reintroduced after processing which can cause foodborne illness.

There is a misconception that food is the main vector for salmonellosis infection in humans. Although food is a potential vector for foodborne infection, it is not the only vector. Therefore, *Salmonella* isolation methods and control methods need to be investigated to better determine the potential threat contaminated food can propose if a correlation is established

between food and salmonellosis infection in humans. Therefore, this review investigates the characteristics of *Salmonella*, pathogen prevalence, detection methods, and control to better understand if there is a direct correlation between animal food and salmonellosis.

SALMONELLA CHARACTERISTICS AND CONCERN

Characteristics.

Salmonella is a common foodborne pathogen. This organism is classified as a Gram-negative, rod-shaped, facultatively anaerobic bacilli that belongs to the *Enterobacteriaceae* family (Giannella,1996; Maciorowski et al., 2006b; D'Aoust, 2007; Dunkley et al., 2008; Jones, 2011; FDA, 2013a). Three types of antigens are used to classify the serovars of *Salmonella* which are the O or somatic antigen, H or flagella antigen, and Vi or the capsular antigen (Dunkley et al., 2008). *Salmonella* is able to survive in various environmental conditions. *Salmonella* can grow in temperatures as low as 2 – 4 °C, but it commonly grows in temperatures ≤ 54 °C (D'Aoust, 1997). *Salmonella* can survive in an environment where pH values range between 4.5 to 9.5, but the most optimal pH for *Salmonella* to grow in is between 6.5 to 7.5 (D'Aoust, 1997). The conditions listed are the most common conditions in which *Salmonella* can grow. However, the survival conditions vary among serovars. For example, *Salmonella* Senftenberg 775W is highly thermal resistant. The thermal death of *Salmonella* is determined by *D*-values (the time it takes the organism to be reduced by 90 % or 1 log) and by z – values (the alteration in temperature that is required to change the *D*-value by 1 log). Ramirez et al. (1997) conducted a study to determine the thermal inactivation rate of *Salmonella* Senftenberg ATCC 43845 and *E. coli* 0157:H7 to known protein markers within ground beef. In this study, the *D*-value of *S.* Senftenberg was 53.0 min at 53 °C and 0.22 min at 68 °C. The z-value for this organism was 6.25 °C. In comparison, the *D* – value for *Salmonella* Typhimurium present in

pasteurized turkey bologna was 278 s at a temperature of 57 °C (McCormick, et al., 2003). Factors that can influence death of organisms include water activity, formulation, food type, and surface type (i.e. stainless steel versus glass; Doyle and Mazzotta, 1999; Awuah et al., 2007; Sperber and the North American Millers' Association Microbiology Working Group, 2007). If adequate temperature and time parameters are not used in addition with proper sanitation and handling procedures, then foodborne infection can occur in humans.

Certain serovars of *Salmonella* have been shown to cause gastroenteritis in humans. There are over 2,500 serovars of *Salmonella*, but non-typhoidal strains *S.* Enteritidis (**SE**) and *S.* Typhimurium (**ST**) represent the main class of serotypes that cause foodborne illness. The most common ways humans may contract *Salmonella* is through improper hand washing or not washing their hands at all, handling or ingesting contaminated food, consuming raw meat and poultry, or from contaminated water, soil, and surfaces (i.e. food contact; Maciorowski et al, 2004; Hendriksen et al., 2011; Andino et al., 2014; Kogut and Arsenault, 2017; FDA, 2019). However, studies have shown that *Salmonella* can be isolated from almost anything. This includes various items and common vectors around the household such as refrigerators, vacuum cleaners, pets, insects, cutting boards, and kitchen sinks (Schutze et al., 1999).

Common symptoms of salmonellosis include diarrhea, fever, nausea, vomiting, headache, and abdominal pain. Symptoms can begin as soon as 8 h after consuming the contaminated food and can last for as long as 5 d (Maciorowski et al, 2004; FDA, 2013a). *Salmonella* infects the body by attaching to cells in the small intestine. Next, the enterocytes invade, and the infected epithelial cells burst into the intestinal lumen which results in the intestinal tract losing its absorbing capabilities (Meerburg and Kijlstra, 2007). Individuals that are commonly infected with salmonellosis are those that are immunocompromised, elderly, or children under five yr of

age (Maciorowski, 2004; FDA, 2013a; CDC, 2019a). *Salmonella* is ubiquitous in nature and can survive in various environmental conditions for extended periods of time; therefore, there are many ways in which *Salmonella* can enter a food system (Jones, 2011).

Vectors.

There are various vectors that can transmit *Salmonella* to animal food such as wild birds, rats, mice, beetles, flies, and cockroaches. These animals are a reservoir for *Salmonella* transmission and can carry bacteria within their intestinal tracts while remaining asymptomatic to the infection (Maciorowski et al, 2004; Meerburg and Kijlstra, 2007; Koyuncu et al., 2013). Pests and wild animals are attracted to foodstuffs that have been spilled, as well as areas that provide available water and adequate shelter. Therefore, it is necessary to reduce food and water spillage to deter the attraction of these animals. By following proper pest control procedures, contamination in food, the environment, and animal food manufacturing facilities will be reduced. (Meerburg and Kijlstra, 2007). Rodent, wild bird, and cat feces have been a source of cross-contamination of antibiotic resistant ST Definitive Type 104L on cattle and pig farms (Davies and Wray, 1997). Research has shown that rodent droppings and dead mice are the biggest problem on poultry farms. Jones et al. (1991) discovered a *Salmonella* prevalence of 5.3 % from the gastrointestinal tract of mice that were residing in animal food manufacturing facilities and poultry houses. Furthermore, rodents were found to contain 24 % SE on chicken layer farms (Henzler and Optiz, 1992). Since *Salmonella* can be transmitted through the fecal-oral route, chickens will eat the rodent droppings present in their bedding or feeder and become contaminated prior to excretion or shedding of the organism (Meerburg and Kijlstra, 2007). One way that food can become contaminated is through feces shed by animals. There is a concern

that humans will become infected with salmonellosis if they consume a product derived from animals (i.e. meat and eggs) and the animal has consumed contaminated food previously.

Transmission Route.

The pathogen of interest must be ingested to cause foodborne disease. This is achieved through the fecal-oral route (Jay et al., 2005). The route of *Salmonella* infection is thought to begin with contaminated food ingredients and then to animal food. Next, the animal will become infected when it consumes the contaminated ingredients or food but will be asymptomatic to the infection (not showing any clinical signs of infection). Then, the animal products will become contaminated and; finally, humans will become sick with salmonellosis (Riley, 1969). For example, *Salmonella* can exist in the food ingredients and then be transmitted to the mixed food, and finally to live poultry which can potentially produce *Salmonella* positive products such as eggs and meat. Then, these contaminated products will be consumed by humans who can contract salmonellosis (Cox et al., 1986; Butcher and Miles, 1995; Dunkley et al., 2008). Research has shown that one *Salmonella* cell can cause an infection in young chicks (~ 4 d of age); however, it takes several thousand *Salmonella* cells for chicks 6 to 7 weeks of age to become infected with salmonellosis (Himathongkham et al., 1996). Historically, it is important to understand the prevalence of *Salmonella* in food ingredients and complete animal food.

Salmonella has been isolated from complete animal food and animal food ingredients from various cattle production facilities. In a study conducted by Boyer et al (1962), turkeys became infected with *Salmonella* Taksony from the food they were fed. Testing resulted in the *Salmonella* being isolated from the turkey's intestinal tracts as well as from the unopened bags of food that they were fed. Mackenzie and Bains (1976) isolated 17 serotypes from chicken carcasses and were able to correlate 14 (82 %) of the serotypes found in the carcass to food

ingredients. However, it is important to note that the most common serovars isolated from poultry products and animal food (i.e. *Salmonella* Kentucky*)* are non- typhoidal and are usually not capable of causing foodborne illness (Foley et al., 2008). Daragatz et al. (2005) isolated *Salmonella* spp. from 57/1070 (5.3 %) samples of cattle food and food ingredients, but none of the *Salmonella* spp. isolated caused enteric disease in cattle. Although animals may contract salmonellosis, performance standards are established for production animals (i.e. poultry) in the processing plant to mitigate *Salmonella* contamination. In addition, proper handling and cooking guidelines should be followed subsequently in homes to prevent contamination and ultimately foodborne illness.

Foodborne disease has been defined as "an incident in which two or more persons experienced a similar illness after ingestion of a common food, and the epidemiologic analysis implicated a food as the source of illness;" however, foodborne illness is more subjective and the cause is not directly linked to the food (Bean and Griffin, 1990). In the U.S., animal food has not been found to be contaminated with *Salmonella* at a high prevalence. Of the strains isolated from food, the majority are non- typhoidal (Magossi et al., 2019). There is no demonstrated direct link between contaminated animal food or animals fed contaminated food to the consumption of animal products causing salmonellosis in humans (Blank et al., 1996; Franco, 2005; Magossi et al., 2019). Mitigation strategies are used to reduce contamination in food. There is a lingering misconception that animal food is the main vector for transmittance of salmonellosis to humans; thus, this information heightens consumer concerns (Blank et al., 1996; Franco, 2005). There is not a direct link between animal food and salmonellosis in humans although organisms can be isolated from food. A publication from 1973 stated that salmonellosis occurred in humans from consuming poultry contaminated with *Salmonella* Agona at 17 restaurants in Paragould,

Arkansas (Clark et al., 1973). It was stated that the initial outbreak occurred at a poultry farm in Mississippi that utilized Peruvian fishmeal as a protein supplement in the food (Clark et al., 1973). The author inferred that the chicken at the restaurants in Arkansas were contaminated with *S.* Agona but could not have been the primary source of infection due to using proper cooking temperatures (Clark et al., 1973). During the lab analysis, *S.* Agona was recovered from environmental swabs taken in the slaughterhouse and from offal samples that still needed to be rendered. It is important to note that *Salmonella* serovars were isolated from various locations within the poultry-processing and rendering plants, but none of the isolates were *S.* Agona. In addition, *S.* Agona was *not* isolated from the food samples that were collected (Clark et al., 1973; Franco, 2005). This outbreak was not based upon scientific data and was based upon inference. This outbreak highlights the complexity of salmonellosis outbreaks as well as the challenges that occur during investigations to determine the primary source of the outbreak (Franco, 2005). Today, the media continues to use scare tactics with regard to the number of individuals that contract salmonellosis annually. The media also informs the public of foodborne illness outbreaks. Due to the media coverage, consumer awareness has been heightened and the concern of contracting a foodborne disease has increased.

Concern.

Salmonellosis is a major public health concern. *Salmonella* is the second leading cause of foodborne illness following norovirus (Jay et al., 2005). The Centers for Disease Control and Prevention (**CDC**) **estimate** that annually *Salmonella* causes 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths in the United States; however, the actual numbers reported in a survey conducted by the CDC from 2009 – 2014 showed that only 3,158 total outbreaks occurred consisting of 64,044 illnesses, 4,220 hospitalizations and 120 deaths (Starkey, Auburn

University, Auburn, AL, personal communication, 2018; CDC 2019b). The actual reported numbers are a fraction of the estimated numbers. For instance, in 2007, 155,000 cases of salmonellosis were reported to the European Union, but not all cases of foodborne illness are reported (Wales et al., 2010). There are a few reasons to why the reported numbers are lower than the actual numbers. The CDC conducts surveys to maintain accurate surveillance with regard to foodborne pathogen outbreaks. The CDC uses an algorithm to predict the amount of total foodborne illnesses that will occur annually. The estimations are separated by pathogen (i.e. *Salmonella, Campylobacter*). The CDC National *Salmonella* surveillance System has been used since 1962 to collect data regarding laboratory confirmed cases of *Salmonella* from humans in the United States. In 1997, the quality control algorithm was developed known as "cumulative sums" to detect unusual clusters of foodborne outbreaks. This algorithm works effectively to detect unusual outbreaks of Salmonella, but some outbreaks were still missed; therefore, some outbreaks are predicted based upon passed data (Hutwagner et al., 1997). According to the CDC, the number of illnesses, hospitalizations, and deaths are estimated annually to reflect outbreaks from known and unknown sources. Of the known and unknown sources, the number of foodborne illness outbreaks are estimated. There are 31 known pathogens that cause the majority of foodborne illness outbreaks. Again, the estimations are developed using past surveillance and an algorithm is used that accounts for uncertainty (Scallan et al., 2011). Due to the symptoms of the flu being similar to symptoms of salmonellosis, consumers do not always know if they have foodborne illness. Furthermore, if consumers do not remember what they ate, then it is hard to track the symptoms to a particular food. Although the number of foodborne outbreaks for some *Salmonella* serovars (i.e. SE*)* are decreasing, thousands of foodborne outbreaks still occur

annually; thus, the agriculture industry is continuously working to minimize these numbers further (Jackson et al., 2013).

Salmonella can be present in many phases in the farm-to-fork continuum. Production animals such as pigs, chicken, and cattle are reservoirs for organisms including non- Typhi serotypes of *Salmonella enterica*. *Salmonella* has been found in the gastrointestinal tract of animals and can be excreted into the environment (Crump et al., 2002; Maciorowski, 2004; Maciorowski et al., 2006b). Some sources of *Salmonella* in the environment include water, soil and animal feces. *Salmonella* has been isolated and cultured from foods including raw meat, poultry, eggs, milk, and other dairy products (Jones, 2011; FDA, 2019). A survey conducted during 1993 to 1997 observed that 23 % of foodborne illness outbreaks were attributed to eating meat, dairy, and poultry products (Crump et al., 2002). Compared to the survey surveillance from 1993-1997, the surveillance from 1998-2002 displayed that 46 % of the foodborne outbreaks were attributed to "bare-hand contact by handler/worker/preparer." In addition, poultry products, specifically eggs caused the highest outbreaks of SE (9 %). Most of the isolates of *Salmonella* serovars from finished processed animal food and rendered products are attributed to contamination through vectors (i.e. flies, beetles, and mice). In addition, other main reasons for contamination include recontamination from dust in the environment and personnel not practicing proper hygienic procedures (GMPs; Fedorka-Cray et al., 1997). Various sources can contribute to *Salmonella* contamination in animal food; thus, indicating that food is not the only or main vector of causing foodborne infection in humans (Davies and Hinton, 2000).

SALMONELLA PREVALENCE IN ANIMAL FOOD, ANIMAL FOOD FACILITIES, PET FOOD, EGGS AND RENDERING

Animal Food Composition.

Both animal and plant-based proteins are used in animal food. Animal by-product meals are composed of products such as feathers, offal, beaks, and other parts of an animal that are not edible. Plant or vegetable protein consist of soybean meal, oilseed meal, rice bran, and cottonseed, and can contain approximately 10^6 to 10^8 microorganisms (Van Schothorst, 1982; Davies and Hinton, 2000). Mackenzie and Bains (1976) observed that vegetable meals such as sunflower meal can be positive for *Salmonella* at similar frequencies as meat meal. In a survey conducted by the Food and Drug Administration (**FDA**), it was revealed that 56.4 % of the animal protein products and 36 % of the vegetable protein products collected from 124 processors were contaminated with *Salmonella* spp. (Fedorka-Cray et al., 1997). Oilseed meal is a type of vegetable protein that is used as a replacement for ruminant meat and bone meal to reduce the incidence of bovine spongiform encephalopathy in animals. Research has demonstrated a correlation between high concentrations of Japanese oilseed meal (rapeseed or canola meal) and high *Salmonella* colony numbers (Morita et al., 2006). This relationship may be attributed to the ability of fat to protect the *Salmonella* organism from physiological or environmental stresses (Morita et al., 2006). D'Aoust et al. (1997) also observed that *Salmonella* is protected from digestive enzymes when it is encompassed in high-fat foods where *Salmonella* is inside the hydrophobic lipid micelles. Cereal ingredients are a low-risk ingredient

in comparison to ingredients such as oilseed meal (Davies and Wray, 1997). Food has not been frequently contaminated from milled ingredients. This is attributed to the implementation of GMPs, low water activity and the thermal processing parameters that these ingredients undergo prior to becoming a finished product that is consumed. The milling industry conducts a thorough job regarding sanitation procedures, cleaning the grains, and following good agricultural practices to minimize the existence of pathogenic microorganisms as much as possible (Sperber and North American Millers' Association Microbiology Working Group, 2007). Generally, the water activity of cereal grains is low and does not allow for the growth of *Salmonella*; for example, wheat flour has a water activity between 0.40 – 0.60. Generally, *Salmonella* does not favor an environment with lower water activity. Prior to consumption, most cereal grains undergo thermal processing. Through the cooking process, microorganisms introduced into the grains through cross-contamination will be eliminated and will be safe for consumption (Sperber and North American Millers' Association Microbiology Working Group, 2007; Podolak et al., 2010). One example is flour. This cereal grain is "cooked" during the refining process and is used in bakery items (i.e. cake mix) which are cooked prior to being eaten by the consumer. The rare incidences in which cereal grains have been positive for foodborne pathogens have occurred when the grain has not been further cooked such as in raw cookie dough or raw cake mix (Sperber and North American Millers' Association Microbiology Working Group, 2007). In addition, the contamination of cereal grains and other low-moisture foods have occurred when sanitation procedures and GMPs have not been followed (Podolak et al., 2010). It is important to consider that the *Salmonella* serotypes found in food ingredients are not always correlated to those found in processed poultry (Butcher and Miles, 1995). Different serovars can survive in

different conditions; therefore, *Salmonella* can be found in a variety of ingredients as well as in many environments, such as in a food manufacturing facility (Maciorowski et al., 2006c).

Salmonella *Survivability in the Food Production Environment*.

Salmonella can be present in air, litter, and on equipment used in animal food production facilities (Maciorowski, 2004; Maciorowski, 2006a). Due to *Salmonella's* ability to be present in various environments, it is easy to contaminate or re-contaminate animal food with organisms after the finished food has been manufactured. *Salmonella* survivability is dependent on the amount of moisture in the food and the temperature at which it is processed and stored. In food, *Salmonella* can survive up to 16 months when the storage temperature is ~ 25 °C and 18 months at 11 °C. *Salmonella* is also able to survive in animal and human foods with low water activity. This is especially a concern in dried foods because the foods have already undergone thermal processing steps (Juven et al., 1984; Gabis, 1991; Maciorowski et al, 2004). The survivability of *Salmonella* in dry products may be attributed to cross-contamination after thermal processing has occurred as well as thermo-resistant strains of *Salmonella* (i.e. *Salmonella* Senftenberg; Riemann, 1968; Liu et al., 1969; Goepfert, 1970; Corry, 1973; Amado et al., 2013). A survey was conducted by the World Health Organization to assess the factors that contributed to the existence of pathogenic microorganisms in finished food. The data demonstrated that cross-contamination could be attributed to hygiene practices being insufficient at 1.6 %, cross-contamination at 3.6 %, storing or processing ingredients in locations that are inadequate at 4.2 %, contaminated equipment at 5.7 %, and lastly, contamination present by personnel at 9.2 % (World Health Organization, 1995). This data displays that the main contributor of cross-contamination is from personnel; therefore, further highlighting that proper hygienic procedures *must* be implemented to reduce the existence of microorganisms in the plant environment and in

food. *Salmonella* can be present in niches where water mixes with finished product. In the food processing environment, water can be present on the inside of conveyers, leaky roofs, steam valves, and in finished product loading and unloading stations (Gabis, 1991; Maciorowski et al, 2004). If these areas are neglected during cleaning procedures, biofilms can form which will make *Salmonella* difficult to eradicate (Jones, 2011). In addition to the food processing environment, *Salmonella* can be found in egg processing facilities.

Eggs.

Salmonella Enteritidis is one of the main serovars isolated from contaminated eggs, layers, and the environment. Symptoms of salmonellosis from SE include abdominal cramps, diarrhea, and fever (Trampel et al., 2014). This egg rule requires facilities to mandate pathogen contamination programs (Laestadius et al., 2012). The FDA established a final egg rule (21 Code of Federal Regulation Parts 16 and 118) in 2009 that explained the mitigation strategies that need to be followed in order to prevent the contamination of SE at egg processing plants during production, storage, and transportation. The egg rule states that biosecurity programs must be followed to prevent contamination. For example, companies can implement cGMPs to prohibit personnel from walking to different farms and to prevent the entry of wild animals and pests in the farm and food manufacturing facilities (FDA, 2009). A major outbreak occurred across 11 states due to contaminated shell eggs with SE. Most of the contamination (15/29 restaurants) was linked back to Wright County Egg. The FDA conducted a survey of the facilities by collecting approximately 600 samples from each farm (i.e. animal food facility, surfaces in the farm, etc.; CDC, 2010). This recall was the largest egg recall that had occurred. It is important to recognize that the FDA egg rule was not in place prior to the recall. It was originally thought that *Salmonella* was only present in the poultry food; however, the owner of Wright County Egg, "Jack" DeCoster was not following proper guidelines at multiple food facilities with regard to

food safety. Upon inspection of these facilities, the FDA found openings in the facility's infrastructure which allowed live rodents, maggots, flies, and birds to enter the egg processing facilities. The rendering industry was criticized for this particular *Salmonella* outbreak due to the inclusion of meat and bone meal in the food formulation; however, after investigation reports were released, it was found that the premises were infected with mice and flies and personnel were not following proper biosecurity guidelines (Caparella, 2010). In addition to the contamination of eggs, the high incidence of pests and disregard for biosecurity rules caused areas in the production facility to be contaminated (Laestadius et al., 2012). This investigation suggests that the best way to prevent *Salmonella* prevalence in poultry farms, processing, and manufacturing facilities is to practice proper biosecurity measures (Trampel et al., 2014).

Rendering.

The goal of rendering is to provide safe rendered products (i.e. protein and fat) to formulate food for production animals and pet food (Franco, 2006). During rendering, by-products are cooked at approximately 100 °C with the addition of water to eliminate the presence of microorganisms and to separate tallow and fat (Riley, 1969; Van Schothorst, 1982). However, these by-products may sometimes be re-contaminated when they are handled in their dry state after the cooling process (Gabis, 1991). Meat and bone meal have shown to be a source of *Salmonella* contaminated poultry food (Gabis, 1991). The Animal Protein Producers Industry is one division of the rendering industry that is responsible for biosecurity, testing programs for *Salmonella,* and ensuring that product is not adulterated (Franco, 2006). The Animal Protein Producers Industry also plays a role in training companies on Hazard Analysis Critical Control Point (**HACCP**) principles. By doing so, many companies are choosing to establish either HACCP programs or other food safety programs to assist in providing safe food throughout the

whole rendering process (Franco, 2006). The perspective is that the food continuum begins with safe food ingredients. If the ingredients are safe then the food will be safe. Furthermore, the animals will not have clinical signs of infection and will be healthy. Ultimately, the production of safe food will help ensure that consumers will remain healthy after consuming food products (Franco, 2006). It is essential to provide safe food to animals as well as to humans. For instance, pet owners are responsible for choosing food that provides the highest quality and safety. By providing their animal safe food, pet owners will feel safe and their trust will be increased in food companies if their pets do not contract a foodborne illness. It is important to understand the prevalence of *Salmonella* in pet food to ensure pet food safety.

SALMONELLA IN PET FOOD

Pet Food.

In the United States, there are approximately 43 million households that own dogs and 37.5 million households with cats and this number continues to increase (Behravesh, 2010). Owners are provided with hundreds of choices when picking out food for their pet. Foods that are considered pet food include: fish food, raw meat, treats, varieties of dog and cat food, vitamins, minerals, and animal by-products (FDA, 2013a). The FDA is responsible for regulating pet food, treats, and supplements (Behravesh, 2010). There is a zero-tolerance policy for any *Salmonella* contamination in pet food under the regulation of the Food Safety Modernization Act (Huss et al., 2017). By-products that contain *Salmonella* are considered adulterated under 21 Code of Federal Regulation 500.35 (FDA, 2002). Control measures need to be in place while manufacturing pet food to eliminate *Salmonella* contamination. In pet food manufacturing, kill steps are used as critical control points to eliminate any presence of *Salmonella*. This allows the company to ensure consumers that the food is free of *Salmonella* and that it is safe for their pets

to consume (Warren, 2010). Validation studies should be conducted using surrogate microorganisms to simulate the thermal inactivation of pathogenic organisms to establish effective processing parameters, such as a kill step, to eliminate contamination in pet food (Warren, 2010). Compared to animal food, pet food is regulated more similarly to human food as there is a higher likelihood of human contact with pet food. Humans can become contaminated with *Salmonella* more easily and frequently by handling pet food and interacting with their pet (FDA, 2013a; FDA, 2013b). Furthermore, with pet food, any serotype of *Salmonella* (typhoidal or non- typhoidal) are of concern and the food is considered adulterated when a kill step is not used. However, with food for production animals, only typhoidal (pathogenic) serotypes are of concern (FDA, 2013a).

Symptoms of salmonellosis in dogs include the occurrence of fever, diarrhea, weight loss, and vomiting, but it is not uncommon for dogs to be asymptomatic (Marks and Kather, 2003). Currently, raw pet food is trending. This type of food does not undergo a kill step; therefore, it poses a higher health risk with regard to salmonellosis and listeriosis (FDA, 2018). In addition, it has been advised that owners do not kiss their pet on its mouth after they have consumed raw food in order to prevent salmonellosis occurring zoonotically (LeJeune and Hancock, 2001; FDA, 2018). Contamination can be present on the hide or feathers of an animal from the environment and can eventually reach the carcass during processing steps such as evisceration. If carcasses are not condemned properly and reach consumers, then consumers can develop salmonellosis from mishandling the product. This underscores the need for proper handling, hygienic, and preparation procedures. Awareness of proper control measures on the manufacturing level and handling instructions at home are essential to prevent contamination;

thus, most importantly, prevent foodborne illness (LeJeune and Handcock, 2001; Finley et al., 2006).

Cases of Pet Food and Salmonellosis.

Due to frequent interactions between humans and their pets, there is an increased risk and correlation between contaminated pet food and their owners (Nemser et al., 2014). The first case of salmonellosis in humans that was linked to pet food occurred in 2007 from *Salmonella* Schwarzengrund. This outbreak affected 79 people across 21 states. No deaths occurred; however, of those ill, 39 % was attributed to individuals one year old or younger, 25 % were hospitalized, and the other 32 % acquired bloody diarrhea (Behravesh et al., 2010; CDC, 2007). Environmental samples were collected by the Pennsylvania Department of Health and *Salmonella* Schwarzengrund was cultured from the plant. The United States Department of Agriculture also isolated *Salmonella* Schwarengrund from unopened bags of dry dog food. A voluntary recall was completed by the company (CDC, 2007).

In 2012, a recall occurred with *Salmonella* Infantis present in dry dog food in Canada and the U.S. In this outbreak, there was a total of 20 cases where 18 cases occurred in the U.S. and 2 cases in Canada (CDC, 2012). Samples of unopened dog food bags were linked to the strains that caused salmonellosis in humans and in 2 dogs (CDC, 2012). It is advised that pet owners wash their hands thoroughly after preparing and handling raw or cooked pet food. In addition, young children should be kept away from pet feeding areas to reduce the chances of them putting their hands into their mouths after touching contaminated pet food (CDC, 2007; Nemser et al., 2014). Salmonellosis can occur in humans by ingesting pet food and through contaminated hands or utensils; so, hands, utensils, and kitchen surfaces should be washed thoroughly to prevent the spread of infection (FDA, 2013a; FDA, 2013b).

INCIDENCE OF *SALMONELLA* IN ANIMAL FOOD PRODUCTION FACILITIES

Animal Food Manufacturing.

There are numerous stages throughout the food manufacturing process in which food can become contaminated including: cooling, sieving, sorting, mixing, bagging, transport, and storage. Harvesting, processing, and storage are the 3 main steps in the animal food manufacturing process where animal food can potentially become contaminated with *Salmonella* (Van Schothorst, 1982; Davies and Wray, 1997; Maciorowski, 2004; Maciorowski, 2006a; Maciorowki, 2006c; Amado et al., 2014). Animal food undergoes physical and chemical changes during processing to improve digestibility and prehension. The main goal of pelleting is to improve efficiency (Maciorowski et al., 2006c). Factors that contribute to *Salmonella* being decontaminated in food include the number of *Salmonella* present, thermal resistance of the strain involved, conditioning and pelleting temperature, and heat and moisture transferred in the food (Jones et al, 1991). The goal of food manufacturing is to increase digestibility, uniformity, and handling through physical and chemical processes using machinery (Van Schothorst, 1982; Tabib et al, 1984; Maciorowski et al, 2004).

The pelleting process is composed of three stages: conditioning (mixing steam with meal to create a mash), pelleting, and cooling (Jones, 2011). During conditioning, water is applied in the form of steam to the meal in the conditioner to reach a temperature of around 80 to 85°C for ~ 1 min to create a high moisture mash. At this step in the manufacturing process,

the moisture content of the food is increased by 3 to 4 % (Svihus and Zimonja, 2011). Next, the food will be forced through a pellet die to form a pellet. During the pelleting process, the animal food temperature in the pellet mill increases by another 3 to 10 °C based on the amount of friction when forcing the food through the pellet die (Davies and Hinton, 2000; Svihus and Zimonja, 2011). The pellet quality is dependent on factors including formulation (40 %), conditioning (20 %), drying/cooking (5 %), grinding (20 %), and die specification (15 %) (Fahrenholz, 2012). It has been shown that the raise in temperature produced during food conditioning reduces the majority of vegetative bacteria (Maciorowski et al, 2004). Cox et al. (1986) conducted a study and determined that the conditioning process prior to pelleting is sufficient to kill pathogens present in food. After contaminated food underwent the pelleting process there was an 82 % reduction in *Salmonella* (Jones et al, 1991). Research has also shown that pelleting can reduce colonies of *Salmonella* in food by 50 % (Jones and Richardson, 2004). Mackenzie and Bains (1976) conducted a survey over 3 yr by adding various animal and vegetable ingredients to food to determine if the ingredients were positive for *Salmonella*. The samples collected after pelleting were negative for *Salmonella.* Conditioning time and moisture of the food is crucial in determining the lethality of bacteria during the pelleting process (Jones and Richardson, 2004).

Conditioning is the first step in pelleting. The conditioning process consists of using a conditioner that is comprised of a conveyer with steam injection capabilities. During the conditioning process steam is added to the meal to create a high moisture mash (Riley, 1969). Once the high moisture mash leaves the conditioner, it enters the pellet mill where heat, moisture, and pressure are applied to create an agglomerated pellet. As the pellet leaves the die, the temperature of the pellet is approximately 85 °C. Veldman and others (1995) observed that

Salmonella can be significantly decreased if food is heated between 80 to 85 °C. It has been estimated that by using the conditioning process approximately 10^3 CFU/g of *Salmonella* are killed and the *Salmonella* organism begins to deteriorate at 71 °C (Stott et al, 1975). Pellets must be cooled to reduce heat and moisture. By allowing the pellet to cool and lose moisture, mold and bacteria growth will be reduced (Riley, 1969). In addition to typical pelleting, the Hygieniser® can be utilized after the high moisture mash leaves the conditioner to increase the retention time (**RT**) of the high moisture mash. The Hygieniser® is added during the pelleting process to aid in the reduction of contamination and to produce *Salmonella* free-food (Boney et al, 2018).

General.

Research has shown that food and food ingredients can contain *Salmonella* at levels < 20 colony forming units (**CFU**)/100 g, but *Salmonella* can be found in the animal food processing environment at levels > 10^3 CFU/g (Jones, 2011). It has been estimated that food can take up ~ 1 *Salmonella* organism per 10 to 100 tons of food (Jones and Richardson, 2004). For example, Maciorowski et al. (2004) stated that *Salmonella enterica* can be isolated from various raw ingredients including, oilseed meal, grains, fish meal and other meat by-products.

In the animal food facility, *Salmonella* can be prevalent due to faulty processing and contamination after processing from rodent and wild bird droppings, as well as from dust (Riley, 1969; Koyuncu et al., 2013). Dust serves as a mechanical vector in the animal food manufacturing process. Jones and Richardson (2004) observed that dust accumulation in the manufacturing environment is a major source for contamination in animal food. Nape (1968) observed a linear relationship between the amount of time in which dust was accumulating to the amount of *Salmonella* prevalent in dust samples. The raw ingredient receiving areas contain the

highest amount of dust contamination in comparison to other areas in the animal food facility; therefore, the raw material section of animal food facilities frequently contains the highest amount of *Salmonella* positive samples (Jones and Richardson, 2004). Dust can be created using roller mills, hammer mills, bucket elevator legs, screw conveyers, drag conveyers, and baggers (Jones, 2011). A study conducted in an animal food facility showed that bacterial counts isolated from a dusty animal food facility recovered 20 to 200 organisms per cubic foot of air (Jones and Richardson, 2004). Whyte et al., (2003) observed that 24.2 % of the dust samples collected contained Salmonella. Thus, it is important to manage food manufacturing facilities to control dust accumulation and pathogen prevalence.

It is essential to follow proper sanitation procedures in food manufacturing facilities. For instance, food transportation vehicles must be cleaned to reduce contamination. By following proper hygienic procedures, the rate of ingredient contamination will be reduced. In addition, transportation vehicles will not be a vector for contaminated ingredients and will not cause as much contamination on farms as well as in animal food processing facilities. In a study conducted by Fedorka-Cray et al. (1997), a total of 549 swabs were taken from 25 locations within a grain bin placed onto 22 food vehicles to determine the prevalence of *Salmonella*. Food samples were taken from 17 vehicles to assess the presence of *Salmonella*. Results indicated that 5 of the 22 (22.7 %) food transportation vehicles were positive for *Salmonella*. However, the rate of isolation for the 549 swabs was only 0.7 %. Of the food samples tested, fishmeal, soybean meal, and meat and bone meal were positive for *Salmonella.* The isolates that were cultured from the food samples are not commonly isolated from swine (i.e. *Salmonella* Infantis*, Salmonella* Montevideo, and *Salmonella* Orion) indicating that there may not be a connection from the food to the animal. In this particular study, isolates were not sampled from the swine. Therefore, a

definite correlation could not be established (Fedorka-Cray et al., 1997). If a food sample was found to be positive, it did not mean that the truck that the sample was collected from was positive. Of the vehicles that were sampled, one had visible mold. None of the vehicles were cleaned or disinfected before or after delivering a load of ingredients or food. There is not a direct correlation, but the contaminated food samples could have contributed to the transportation vehicles being contaminated after subsequent loads (Fedorka-Cray et al., 1997). Therefore, proper sanitation methods should be followed to effectively reduce contamination in food transportation vehicles and food samples.

Location.

Different areas of animal food manufacturing facilities can have a higher prevalence of *Salmonella* contamination. For instance, in the preheat areas of animal food manufacturing centers, 11.8 % of the uncooked food ingredients and 33.3 % of the dust samples were positive for *Salmonella* (Whyte et al., 2003). In another study, 18.8 % (15/80 samples) of *Salmonella* positive samples were recovered from preheat areas and 22.6 % of samples were recovered from the post- heat areas. It is important to note that the prevalence in the post- heat areas may have been higher than the pre-heat areas because the food being reprocessed did not reach adequate temperatures. In this particular food manufacturing facility, an alarm would sound when food ingredients were not adequately heated to the pre-requisite temperature. These ingredients may have been contaminated due to not eliminating the potential *Salmonella* prevalence in the food matrix during the kill step. These ingredients were then transported on the post-heating conveyor belt to be reprocessed. Subsequent loads of ingredients could have become contaminated due to the first loads not completing the entire thermal processing control step (Whyte et al., 2003). In the preheat areas, contamination rates are high in food delivery vehicles; for instance, Whyte and

collaborators (2003) found that 57.1 % (4/7 samples) of the food delivery vehicles were positive for *Salmonella*.

Research has been conducted in Danish food manufacturing facilities to assess the locations where *Salmonella* could be present. It was discovered that *Salmonella* exists where moisture condensation is present, such as in the pellet cooler (Israelsen et al, 1996; Richardson, 2000). Davies and Wray (1997) surveyed 10 food manufacturing facilities in Great Britain to determine the prevalence of *Salmonella*. The highest amount of *Salmonella* contamination was isolated from the pellet cooler at 85.7 %. Jones et al. (1991) found that 85 % of food sampled from the cooler was contaminated with *Salmonella.* Food that has been heat treated can still be susceptible to recontamination in the pellet cooler. Thermal processing is one mitigation strategy that is used to control and prevent *Salmonella* in food (Amado et al., 2013).

In addition to various locations, *Salmonella* may be present during different seasons. Israelsen and collaborators (1996) suggest that contamination rates can vary during different seasons. For example, research has shown that contamination can be more prevalent in the cool seasons in comparison to the warm seasons due to the increase in condensation and change in humidity (Israelsen et al., 1996). The fluctuation in humidity can affect the quality of pellets and can potentially create more moisture prevalence in the food. The pelleting process is utilized to improve feed conversion ratio, decrease feed wastage, improve pellet quality, and reduce pathogen prevalence. It is important to understand how microorganisms can be prevalent during the pelleting process and how to control these organisms to produce safe food for animals.

Salmonella *Prevalence During Pelleting.*

Broilers are fed pelleted food to decrease the energy being utilized when consuming large amounts of food in a short amount of time (Riley, 1969). A study was conducted to assess the

amount of time that it took turkeys and chickens to consume pellets versus mash over a 12 h period (g/bird/12 h). Results showed that when mash was fed, turkeys spent 8.5 times as much time eating mash diets compared to pelleted food and chickens spent 3 times as long, respectively. Pelleting is recommended as an intervention strategy during food manufacturing to reduce and optimally eliminate pathogens present in various production animal foods (i.e. broiler, swine, etc.; Bhatia and McNabb, 1980; Fedorka-Cray et al., 1997; Richardson, 2000). *Salmonella* is prevalent in animal food; however, not all serotypes are pathogenic. A survey was conducted in the Netherlands from 1990 to 1991 where 21 % of the poultry meal was found to be contaminated with *Salmonella,* but it did not contain pathogenic strains such as SE or ST. Surveys were also conducted in the U. S. where 16 % of the food was contaminated (Davies and Wray, 1997). In an Ontario animal food facility, 4.3 % of 93 finished pelleted broiler foods were contaminated with *Salmonella* (Hacking, 1978). A survey was conducted in a Dutch animal food facility that manufactured poultry food and it was found that meal for layers was more contaminated (21 %) than the pellets (1.4 %). This is a total reduction of 93.33 % between the meal food and the pelleted food (Veldman et al, 1995). Bhatia and McNabb (1980) found that the food samples collected from farms A, B, C, D, and E were all negative at the 3- and 6-week collection period and only one sample from Farm C was positive at d 1.

Food is sampled at various steps throughout the production line to identify prime areas of contamination (Davies and Wray, 1997). Aggregate samples can be taken from the food entry point of the cooler to confirm contamination from spillage around the cooler (Davies and Wray, 1997). *Salmonella* is capable of being prevalent in raw ingredients, food contact surfaces, as well as machinery within the food production facility. In order to fully understand the prevalence of

Salmonella within the farm and food manufacturing facilities, it is essential to understand how to enumerate bacteria utilizing various methods.

TESTING FOR *SALMONELLA* IN ANIMAL FOOD

Traditionally, cultural growth-based methods are used in combination with selective media to isolate and enumerate *Salmonella* in animal food (Maciorowski et al, 2004). Instantaneous testing cannot be implemented to test for *Salmonella* directly in an animal food manufacturing environment due to the majority of *Salmonella* testing techniques taking approximately 2 d to obtain results. This could propose a possible issue if the results are positive (Davies and Hinton, 2000). In production animal food facilities, there is not a hold and release program; therefore, the ingredients that were used for the finished food will enter the food chain at a rapid rate (Davies and Hinton, 2000). It is difficult to isolate *Salmonella* because it is usually present in low quantities or is unevenly distributed throughout the batch of food (Maciorowski et al, 2004). Other problems include the load sizes of the receiving ingredients being large. This is a problem because in order to test a representative number of samples, numerous samples must be taken (Davies and Wray, 1997; Richardson, 2000). Due to the difficulty enumerating *Salmonella*, non- *Salmonella* indicator organisms such as *Enterobacteriaceae* and *Escherichia coli* (*E. coli*) can be used to detect *Salmonella* in animal food (Maciorowski et al, 2004). Jones (2011) reported that if *Enterobacteriaceae* counts are $\geq 10^4$ CFU/g in unprocessed foods or are present in processed foods at $\geq 10^2$ CFU/g then the *Enterobacteriaceae* may be a reliable indicator to show that the food contains *Salmonella*. There are various methods that can be used to test for prevalence of *Salmonella* in food, but these testing methods have not been adapted for the fast-paced animal food facility environment.

METHODS TO REDUCE *SALMONELLA* CONTAMINATION IN ANIMAL FOOD

Overview.

Salmonella inactivation is dependent on numerous factors such as the presence of oxygen, moisture, and temperature used during processing and storage (Van Schothorst, 1982; Richardson, 2000). One approach that can be used to control *Salmonella* growth is hurdle technology. Hurdle technology is a method that is utilized to ensure food safety and quality in foods by utilizing multiple preservation methods synergistically which are known as "hurdles" (Leistner, 2000). With regard to animal food, one example of hurdle technology that can be utilized to provide food safety and quality are thermal processing and chemical addition together (Jones, 2011). The control of *Salmonella* can be broken down into three categories: preventing contamination from entering the facility, reducing microbial multiplication within the plant environment, and developing methods and procedures to kill the pathogen (Jones, 2011). To reduce contamination in the animal food facility as well as on the farm level, it has been recommended that pellet mills maintain high standards with regard to hygiene. Some of these procedures include cleaning steam traps and transition guards, and properly sweeping the floor (Jones and Ricke, 1994). Dust needs to be controlled to reduce the potential for contamination of animal food (Maciorowski et al., 2004; Maciorowski et al, 2006a). Lastly, storage bins should be cleaned and emptied regularly to minimize dust accumulation and contamination in food (Davies and Hinton, 2000). Thermal processing is one way in which bacteria can be stunted and growth can be controlled. Another way in which contaminants can be mitigated in animal food is

through the application of chemicals. Research has suggested that *Salmonella* should be reduced by 5 log cycles to be considered a safe food for distribution and consumption (Liu et al., 1969).

Chemical Use.

Thermal processing has been shown to be effective in reducing *Salmonella* prevalence in food; however, some of the strains are highly thermo-tolerant and recontamination can occur after processing. To assist in the mitigation of pathogens, chemical additives are used. (Koyuncu et al., 2013; Jones et al, 2018). Organic acids disrupt the homeostasis of the cell membrane by altering the pH regulation and, thus, injuring cells causing death of bacteria (Wales et al., 2010). There are several factors that can determine the efficacy of chemical compounds such as the strain, prevalence of *Salmonella* in the substrate, the concentration (i.e. ppm) of the chemical additive, and lastly, the time of contact between *Salmonella* and the chemical being utilized (FDA, 2002). Some chemical compounds that can be added to food include organic acids, such as propionic and formic acid. These acids can be applied to animal food to reduce or eliminate *Salmonella* prevalence. However, organic acids are not 100 % effective in killing *Salmonella* due to the organism not being evenly distributed throughout the food (Maciorowski et al, 2004; Boney et al, 2018). Only 0.2 to 2 % of organic acids are applied to food using the coater; however, the efficacy of the organic acids is highly variable. It can take numerous d for organic acids to work properly and destroy many bacteria. The effectiveness of organic acids depends on many factors such as the level of contamination, the synergistic effects of blending acids, physical form of the compound, moisture present in the food, the rate of addition, chemical formula of the product, and diet composition (Jones, 2011; Koyuncu et al., 2013). If the organic acids are being used for several d, then this process will become costly as the equipment in the food manufacturing facility can become corroded. Another disadvantage of using organic acids

is that the animal must metabolize or excrete the acids without absorption occurring to prevent residues in the animal products (meat, milk, or eggs; Jones, 2011). Although it is volatile, formaldehyde is a common chemical additive that is used to mitigate contamination in animal food (Davies and Hinton, 2000; Cochrane et al., 2016a; Jones, 2018). Ruano and others (2001) observed that by applying formaldehyde to food for 3 h or more, bacterial, fungal, and viral pathogens were eliminated (Jones, 2018). Formaldehyde and medium-chain fatty acids are important additives to consider for *Salmonella* mitigation in food. These compounds are capable of eliminating *Salmonella* when there are low levels of microorganisms present in the food, as well as for preventing recontamination during post-pelleting (Cochrane et al., 2016a; Jones, 2018). Cochrane et al. (2016b) observed that organic acids were sufficient in killing *S.* Typhimurium ATCC 14028 in pelleted food as well as in preventing recontamination following processing. Furthermore, formic acid has been shown to be effective in reducing *Enterobacteriaceae* when compared to acetic, lactic, and propionic acid at 20 °C and a moisture content of 13 to 15 % in the food (Van Schothorst, 1982). As stated, there are some benefits to adding chemicals to food in order to mitigate *Salmonella*; however, there are some basic principles to follow in order to control entrance of pathogens into the food manufacturing environment such as, pest control, biosecurity, GMPs, and HACCP.

Pest Control.

It is essential to decontaminate farms to reduce the possibility of *Salmonella* prevalence within the environment and within food. Contamination can be reduced by establishing pest control procedures. Pests can be eliminated by inspecting and improving farm buildings, and by removing old materials that are no longer in use (Meerburg and Kijlstra, 2007). Furthermore, it is essential to depopulate pests that serve as reservoirs for *Salmonella* around the premise of the

food manufacturing environment. In addition to pest control, biosecurity measures should be implemented to reduce contamination.

Biosecurity.

Biosecurity measures such as disinfecting boots and clothes, as well as maintaining who is arriving and leaving the facility will better ensure that the amount of *Salmonella* is decreased (Meerburg and Kijlstra, 2007). Pans with disinfectant should be used to assist in the mitigation of contamination and to reduce cross-contamination among areas of the manufacturing facility as well the farm (United States Agricultural Research Service: Animal Health Division, 1966). If required, employees should shower-in and shower-out to reduce cross-contamination between facilities (United States Agricultural Research Service: Animal Health Division, 1966).

Hazard Analysis Critical Control Point.

In Europe, legislation requires that a HACCP plan is used in all animal food manufacturing facilities to eliminate biological hazards including pathogens such as *Salmonella* (Bucher et al., 2007). A hazard analysis should be conducted where critical control points are identified in order to reduce the potential of *Salmonella* from entering the plant and negatively effecting animal food and potentially consumers (Butcher and Miles, 1995). The first step in a hazard analysis is to formulate a flow diagram to easily identify ingredients used and process steps such as receiving, processing, storage, packaging, loading, and delivery (Cochrane et al., 2016a). The FDA defines a hazard as "a biological, chemical, or physical agent that has the potential to cause illness or injury in humans or animals (Cochrane et al., 2016a)." By conducting a risk assessment of the entire food facility, problem areas will be identified, and strategies can be formulated to reduce microbiological biofilms and aggregate throughout the equipment. Equipment should also be tested frequently to ensure that it is working properly and

is not deviating from the set process parameters (Davies and Hinton, 2000). Incoming raw ingredients should be inspected and provided with a Certificate of Analysis to guarantee that the ingredients meet label requirements and are safe to use; in addition, reputable suppliers should be used when purchasing ingredients (Richardson, 2000).

Transportation vehicles should be routinely inspected and sanitized; in addition, vehicles should be segregated to transport different items such as offal, litter, and raw ingredients (Butcher and Miles, 1995). Facility personnel should inspect ingredient loads for contamination and reject those that do not meet standards. For instance, a survey was conducted at 5 food production facilities to observe the levels of contamination. Researchers found that 67 % of the contamination was from animal by-products, but this could have been avoided by visually inspecting the meal or conducting tests to determine pathogen prevalence. In this study, the meat meal was contaminated prior to arriving at the food manufacturing facility (Nape, 1968; Cochrane et al., 2016a). Haulers should understand what cleaning procedures are being used on their vehicles and should be aware of what ingredients were included in the 3 prior loads delivered (Jones, 2011). To reduce contamination further, raw material receiving areas and processing areas should be divided and personnel should only work in one area to reduce cross-contamination. Drivers should also be required to stay inside their vehicles to minimize contamination. If drivers need to get out of their vehicle, they should be required to wear sterile shoe covers (United States Agricultural Research Service: Animal Health Division, 1966; Cochrane et al., 2016a). Another way to reduce cross-contamination is to assign colored tools to each area to eliminate mixing of tools in dirty and clean areas (United States Agricultural Research Service: Animal Health Division, 1966). Dust can also be greatly reduced by routinely vacuuming (United States Agricultural Research Service: Animal Health Division, 1966;

Richardson, 2000). In addition to implementing procedures throughout the animal production facility, personnel must be informed on proper biosecurity procedures.

Personnel.

It is essential that facility personnel are properly trained on handling techniques, aseptic sampling, and GMPs to ensure that guidelines are being followed to reduce *Salmonella* contamination in animal food and pet food (Huss, 2015). One of the easiest ways that contamination can be eliminated is if personnel wash their hands thoroughly, wear gloves when needed, and use sterile scoops when sampling (United States Agricultural Research Service: Animal Health Division, 1966). If *Salmonella* or other contaminants are found in the environment or in the food, corrective actions with regard to sanitation procedures should be used to eliminate contamination (United States Agricultural Research Service: Animal Health Division, 1966).

Sampling Procedures.

When taking samples from a food or rendering plant, it is crucial to practice aseptic technique to significantly reduce contamination (Jones, 2011). Jones (2011) observed that when facility personnel were not using aseptic technique, the samples were 43.75 % positive. Only 7.32 % of the samples were positive for *Salmonella* when researchers used aseptic technique to collect samples. This study shows that aseptic technique is necessary to obtain accurate results on actual contamination rates (Jones, 2011). Ingredients can become contaminated through processing. It is not uncommon for ingredients to be handled 10 to 15 times before the finished product is shipped to the customer. Control procedures should be followed, and aseptic technique should be utilized at all times in the food processing environment to reduce contamination (Jones, 2011).

Food Withdrawal.

To reduce *Salmonella* contamination in poultry prior to slaughter, food withdrawal procedures are used. The optimal time for food withdrawal with poultry is 8 to 12 h prior to slaughter. The goal of this process is to reduce fecal contamination from occurring on the carcasses during processing. The United States Department of Agriculture has a zero tolerance for fecal contamination and the birds must be reprocessed, trimmed, or condemned. In addition to fecal contamination, there can be contamination in the food. Mackenzie and Baines (1976) found that food or food ingredients were responsible for 82 % of *Salmonella* present on broiler carcasses. By establishing food withdrawal times, there will be smaller amounts of food in the bird's intestinal tract. A survey conducted from 2003 to 2005 in U. S. poultry slaughterhouses, demonstrated that 20 % of the broiler chicken carcasses were contaminated with strains of *Salmonella* that are harmful (Meerburg and Kijlstra, 2007). In order to mitigate *Salmonella* contamination on poultry carcasses, food withdrawal strategies are used concurrently with the United State Department of Agriculture - Food Safety and Inspection Service. This governmental agency has established performance standards as a regulatory mandate to reduce pathogens where the pathogen tolerance in the performance standards continues to decrease annually (Franco, 2005).

Handling and Cooking Procedures.

Consumers of animal products should follow handling and cooking guidelines to restrict potential contamination of the product and to prevent sickness. It is known that humans can become infected once they consume contaminated meat, eggs, or raw produce that is contaminated with feces or are undercooked (Crump et al., 2002). Salmonellosis can also occur

if humans handle contaminated food such as raw pet food, ingest bacteria or by touching contaminated surfaces (FDA, 2019).

Regulations.

It is the FDA's responsibility to ensure that the food has been labeled properly, that it is safe, and that the food is free of any human health hazards (Crump et al., 2002). The FDA Compliance Policy Guide (Sec 690.800) "*Salmonella* in Food for Animals", states that food should be seized if one or more of the food samples contain *Salmonella* and if the food will not be irradiated to eliminate *Salmonella* or if *Salmonella* has the potential to be transmitted to humans if they ingest or handle the food ingredient, or if this strain of *Salmonella* is pathogenic and can harm the animal being fed (FDA, 2013a)." According to the Food Drug & Cosmetic Act, animal food or pet food that contains foodborne pathogens (i.e. *Salmonella)* and have the potential to cause disease and illness in animals are considered adulterated (Franco, 2005). The FDA only considers animal food to be adulterated when it is contaminated with a *Salmonella* serotype that is pathogenic to the animal and it is being fed. Furthermore, the food that will be fed to an animal will not undergo a kill step (FDA, 2013a; Magossi et al., 2019). Common serotypes that are considered pathogenic to production animals include *Salmonella* Gallinarum, *Salmonella* Pullorum and SE in poultry food, *Salmonella* Dublin and Newport in dairy and beef cattle food and *Salmonella* Choleraesuis in swine food (Fedorka-Cray et al., 1997; FDA, 2013).

Surveys.

Surveys are used to gather necessary information about foodborne illness occurrence and prevalence of *Salmonella* in animal food from different locations in the food facility. Surveys have shown that the prevalence of *Salmonella* in animal food has been decreasing while the salmonellosis cases in humans has not decreased (Gareis, 1995). Researchers have been trying to

control and eliminate *Salmonella* in poultry products for more than 25 years (Jones et al, 1991). It is important to recognize that the prevalence of *Salmonella* has been decreasing in animal food. The FDA conducted a survey from 2002 to 2006 in U.S. food facilities where animal ingredients had a contamination rate of 30.9 %, but in 2007 to 2009 was only 19.4 % (Li et al, 2012). From 2002 to 2009, 12.5 % of food and food ingredient samples were found to contain *Salmonella* (Li et al., 2012). A survey was conducted to assess the prevalence of *Salmonella* in various food facilities, as well as to determine which locations/objects had the highest isolation of the organism. Magossi et al. (2019) observed that 92.5 % of worker's shoes, 81.8 % of the finished product bin boot, 80.6 % of the ingredient pit, and 80.6 % of the floor dust in the receiving area was present with *Salmonella.* The lowest prevalence of *Salmonella* was in the fat tank inlet (20 %,), 33.3 % in the exterior pellet mill, and 41.7 % in the finished food (Magossi et al., 2019). It was also observed that the *Salmonella* prevalence decreased as the food went from initial processing to the finished product (Magossi et al., 2019).

In 2000, the World Health Organization developed the World Health Organization Global Foodborne Infections Network to detect, control, and prevent foodborne infections through laboratory testing and surveys. From the initial survey, it was found that SE was the most common isolated serovar in North America, followed by ST (Hendriksen et al., 2011). It is important to recognize that overall, the isolation of both SE and ST prevalence has decreased. SE has decreased in developing countries from 73.9 % in 2001 to 55 % in 2007 and ST has decreased in developed countries from 26.4 % in 2001 and 18.8 % in 2007 (Hendriksen et al., 2011).

IMPLICATIONS

Thermal processing is utilized to improve food quality and to ensure food safety. The goal of thermal processing is to greatly reduce the microorganism of interest or targeted organisms prevalent below detectable levels in order to prevent foodborne illness from occurring in humans and animals while maintaining food product integrity (Awuah et al., 2007). Awuah et al. (2007) states that in order to effectively reduce the pathogen of interest utilizing thermal processing methodologies, factors should be considered including packaging, pH, type of food, thermal resistance, and salt content within the food matric.

A previous study was conducted to: 1) evaluate *Enterococcus faecium* as a surrogate microorganism to measure the resistance of *Salmonella* in the animal food manufacturing environment when utilizing 3 conditioning temperatures (**CT**) and 3 RT in the Hygieniser®; 2) assess the effect of the Hygieniser® as an newer addition to the conditioning process; 3) evaluate the quality of food with regard to the prevalence of heat labile nutrients such as amino acids and vitamins present after the pelleting process, and 4) assess the amount of starch gelatinization (**SG**) that was attained using combinations of 3 CT and 3 RT. *E. faecium* is an organism that is commonly found in the GI tract of mammalian species. *E. faecium* has been validated as an appropriate surrogate in ground beef (Ma et al., 2007), carbohydrate- protein meal (Bianchini et al., 2014), as well as almonds (Jeong et al., 2011). There has not been much research utilizing *E. faecium* as a surrogate for *Salmonella* species in the animal food manufacturing environment. Therefore, for this study, 9 total treatments were utilized (3 CT and 3 RT) to assess *E. faecium* as a surrogate for *Salmonella.* Unprocessed meal samples were

collected from the bucket elevator as the mixer was emptying and processed high moisture mash samples were collected after the conditioner and Hygieniser®. Lastly, finished pelleted samples were collected exiting the pellet mill prior to entering the pellet chute to the pellet cooler. Each sample was collected in a foil pan and was cooled prior to weighing 25 mg of each sample into Whirl-Pak bags. The samples were then placed on ice and transported from the Charles C. Miller Poultry Research and Education Center to the microbiology lab at Auburn University where microbiological analysis was conducted. For each sample, 225 mL of buffered peptone water was poured into a Whirl-Pak filter bag and the sample was stomached for 2 min. Sample were then serial diluted utilizing 0.1% peptone. To obtain total aerobic bacterial counts, each sample was plated on Tryptic Soy Agar. To determine the total amount of *Enterococcus* present within the food manufacturing facility, m-*Enterococcus* agar was utilized as a selective media. With regard to the second objective, the Hygieniser® is a new technology that is utilized during the pelleting process in order to improve the quality of animal food by extending the time that the high moisture mash is retained once it leaves the conditioner. In addition to quality, this equipment is utilized to produce animal food that is free of *Salmonella* or other pathogens. Therefore, we wanted to assess the addition of the Hygieniser® to the conditioning process when compared to literature that only utilizes the traditional conditioning process. Results for objective 1 and 2 indicated that the total bacteria count was consistent, if not higher, for all of the CT and RT combinations. *Bacillus* spp. are bacteria that can withstand thermal processing, which may explain why there was a high prevalence of bacteria counts on the TSA. At all conditioning temperature and retention time combinations, *E. faecium* was reduced. When samples were conditioned at 167 °F, the *E. faecium* was reduced by approximately 2 log CFU/g. It was observed that samples collected after the Hygieniser® were reduced by another log when

compared to the conditioned samples at 167 °F and samples collected after pelleting had *E. faecium* reduced by another 1-1.5 log. Samples conditioned at 185 and 203 °F did not have any detectable counts of *E. faecium*; thus, indicating that the bacteria were reduced by more than 5 logs. It is important to note that few samples collected after the pellet mill was declared negative but were able to be accounted for after enrichment. The two CT and RT combinations where this occurred was at 185 °F and 203 °F with a RT of 240 s for both temperatures (E, Monu, unpublished data). This study underscores the importance of validating surrogate organisms in different food matrices and in different thermal processing conditions to truly understand how organisms behave, the prevalence of organisms, and how to control the microorganisms in order to produce safe food for animals and humans.

During food manufacturing, food ingredients can undergo physical changes such as bulk density and particle size and chemical changes such as SG (Moritz et al., 2002b; Moritz et al., 2005). Vitamins and amino acids are regarded as heat labile nutrients in the diet and may undergo alteration during the food manufacturing process. Research has shown that vitamins are generally stable during the food manufacturing process, but vitamins such as A, K and C are broken down easily (Broz et al., 1997). Diets are formulated with high amounts of corn which indicates that the diets contain high amounts of starch (Moritz et al., 2005). Starch is the main energy source in broiler diets. When heat and water are added during the food manufacturing process, the gelatinization of starches aid in binding and therefore, aid in creating an agglomerated pellet. It is thought that by improving the accessibility to enzymes in the starch granule, that digestibility is improved. Zhu et al. (2016) utilized total starch (AOAC 996.11; AACC 76.13 and glucoamylase; AACC 76.11) and SG methodologies (differential scanning calorimetry and glucoamylase AACC 76.11) to assess total starch and SG methodologies in a

multi-component pelleted swine food. They used 2 CT at 77 and 88 °C and retention times at 15, 30 and 60 s. The study conducted by Zhu et al. (2016) utilized traditional CT and RT combinations. A study was conducted utilizing 2 traditional CT at 75 and 85 °C and 1 non-traditional CT at 95 °C. The RT that were utilized were 80, 160 and 240 s which is much longer than the times utilized in industry. Lastly, the present study utilized a Hygieniser®. This is a newer technology that is being utilized in food manufacturing, but Zhu et al. (2016) did not utilize this technology for their study. For this study, SG and total starch were assessed for a commercial corn-soybean meal-based broiler food manufactured at 3 CT and 3 RT combinations. SG was assessed in the food using differential scanning calorimetry and AOAC 996.11 was utilized to obtain total starch (%) in cereal products. There have not been many studies conducted regarding SG and total starch in multi-component food processed utilizing various CT and RT combinations; therefore, by conducting this study, new information can be obtained to better understand if the combinations being utilized are gelatinizing food and if this is the primary reason to why animal performance (i.e. energy utilization) is improved when pellets are fed.

CONCLUSION

Salmonella is a ubiquitous organism that is typically present in the environment (i.e. soil), air, dust, and in raw meat and poultry; therefore, it is able to adhere to various surfaces and equipment within the food manufacturing facility as well as in food products, such as animal food. Mitigation strategies are utilized such as thermal processing and chemical application to reduce the prevalence of pathogens in the finished food product. GMPs, aseptic technique, and biosecurity are also essential practices that need to be utilized to decrease pathogen prevalence. Although, food can be contaminated, it is not the initial source of foodborne illness to humans.

Raw ingredients are contaminated prior to arriving at the manufacturing facility or finished food is contaminated by utilizing improper cooking instructions, handling cleaning procedures, and by not thoroughly cleaning and maintaining the plant. Sources that can contaminate food include dust, moisture, and vectors such as flies, cockroaches, or mice. Over the last decade, food facility managers and personnel have become more aware of mitigation strategies that should be implemented within the food processing facility due to the implementation of the Food Safety Modernization Act. With this regulatory addition, the prevalence of pathogens in the food manufacturing facility have decreased. Personnel working at food processing plants will continue to implement proper practices to reduce pathogen prevalence and ultimately foodborne outbreaks and illness in consumers.

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CHAPTER II: EVALUATION OF DSC TO ASSESS STARCH GELATINIZATION AND AOAC 996.11 TO ASSESS TOTAL STARCH CONTENT IN MULTI COMPONENT ANIMAL FOOD UTILIZING RETENTION TIMES AND CONDITIONING TEMPERATURE

INTRODUCTION

Animal food is formulated with ingredients such as carbohydrates, proteins, fats, vitamins, and minerals. Carbohydrates typically make up the largest portion of the diet formulation. In most animal diets, starch provides the highest source of energy to the animal (> 50 % Apparent Metabolizable Energy; Kronfeld and Van Soest, 1976; Moran Jr.,1982; Weurding et al., 2001; Batal and Parsons, 2004; Zhang et al., 2006a; Zimonja and Svihus, 2009; Zhu et al., 2016; Ranathunga et al., 2017; McCleary et al., 2019). Corn and soybean meal is used to formulate swine and broiler diets. The starch present within the corn is capable of gelatinizing and increasing energy digestibility in swine and broilers (Zhu et al, 2016). Dietary starch is defined as an "alpha-linked glucose carbohydrates of or derived from plants, animals, and microbes from which glucose is released after gelatinization through the use of purified α-amylase and amyloglucosideases that are specifically active only on α-(1,4) and α-(1,6) linkages (McCleary et al., 2019)." When formulating diets, it is important to consider the amount of dietary starch included in the diet as it can influence animal performance (Hall, 2009). During food manufacturing, food undergoes thermal and mechanical processes to improve nutrient availability, digestibility, and efficiency with regard to energy and time savings

(Leeson and Summers, 2005; Hemmingsen et al., 2008; Lewis et al., 2015 a, b). Jensen et al., (1962) conducted a study where they observed the average amount of time turkeys and chicken spent eating in a 12 h time interval per d, as well as the average amount of food that was consumed (g/bird/12 h). Results from this study indicated that when mash was fed, turkeys spent 8.5 times as much eating mash diets compared to pelleted. The chicken spent 3 times as long consuming the mash diets compared to the pelleted food. This highlights the importance of formulating a pellet that is highly durable in order for the bird to perform optimally and to reduce energy waste (Leeson and Summers, 2005). Furthermore, while high pellet quality shortens consumption time, it has been speculated that starch gelatinization (**SG**) can improve digestibility. During SG, the starch granule ruptures and the crystalline arrangement within the granule becomes disorganized. The glycosidic linkages are exposed and are more available to enzymatic breakdown by α- amylase from the pancreas (Björk and Asp, 1983; Moritz et al., 2002a; Moritz et al., 2005; Htoon et al., 2009; Zimonja and Svihus, 2009; and Svihus, 2014). Zhang et al. (2006b) defines resistant starch as starch that is incapable of being digested in the small intestine but is fermented in the large intestine. Resistant starch is divided into 4 categories: RS_1, RS_2, RS_3, and RS_4 (Haralampu, 2000; Htoon et al., 2009). Respectively, the starch types represent starch that cannot be reached during digestion, un- gelatinized starch, retrograded starch, and chemically modified starch (Haralampu, 2000). Two other types of starch include rapidly digestible starch which can be digested in 20 mins or less and slowly digestible starch which can be digested between 20 -120 mins (Zhang et al., 2006b). Food undergoes physical changes such as bulk density and particle size and chemical changes such as Maillard reaction or SG when manufactured (Plavnik and Sklan, 1995; Moritz et al., 2005). Pelleted diets are used in poultry and swine feeding to decrease food waste, improve food efficiency and

improve prehension (Briggs et al., 1999; Moritz et al., 2001; Zhu et al., 2016; Massuquetto et al., 2018). Common thermal processes utilized during food manufacturing are conditioning and pelleting; however, recently, Hygieniser® technology is being utilized between the conditioning and pelleting steps to retain the food for a longer period of time to increase pellet quality and provide a *Salmonella* free-food. During conditioning, increasing amounts of moisture are applied in the form of steam (~ 11 to 18 %) to convert a dry meal into a high moisture mash at ~ 80 to 90 °C. The Hygieniser® utilizes a variable speed drive to retain the high moisture mash for up to 4 mins and lastly, the pellet mill uses heat, moisture, and pressure to force the high moisture mash through the pellet die to form an agglomerated pellet (Wellin, 1970; Plavnik and Sklan, 1995; Thomas et al., 1998; Briggs et al., 1999; Gilpin et al., 2002; Leeson and Summers, 2005; Hemmingsen et al., 2008; Lewis et al., 2015a, Zhu et al., 2016; California Pellet Mill Co.). Conditioning temperature (**CT**) and retention time (**RT**) are 2 factors that can influence pellet quality and SG during thermal processing (Zhu et al., 2016). The starch granule is composed of two polysaccharides, amylose and amylopectin, thus creating alternating crystalline and amorphous regions in the starch granule. The crystalline regions of the starch granule are composed of both amylopectin and amylose whereas the amorphous regions comprise of only amylose (Lelievre et al., 1973; Moran Jr., 1982; Ratnayake et al., 2006; Zhange et al., 2006b; Svihus, 2014; Schirmer et al., 2015). Amylose is composed of ~1000 α-D-1,4 glycosidic linkages and a small amount of α-D-1,6 glycosidic linkages which creates a linear structure. Amylopectin contains α-D-1,4 glycosidic linkages. Approximately 4000 branched glucose units are formed between the α-1,3 linkages and the α-1,6 linkages, making amylopectin a highly branched structure (Zobel, 1988; Donald, 2001; Svihus, 2014; McCleary; 2019). Generally, the

dry weight of the starch granule is composed of ~ 98 to 99 % amylose and amylopectin (Schirmer et al., 2015).

The conditioning process may improve digestibility by improving nutrient utilization. During conditioning, amylase and amyloglucosidase break down the two starch polysaccharides, amylose, and amylopectin, respectively. The hydrolysis of these two enzymes aid in increasing the digestibility of carbohydrates during digestion (Massuquetto et al., 2018). To form a good pellet, the optimal moisture content should be ~ 15 to 18 % (Leeson and Summers, 2005).

One of the chemical changes that food can undergo is SG. SG is defined as an order-to-disorder phase transition; as heat and excess water are added to the system, the starch granules swell, and the primary structure of the granule is disrupted. As the starch granule dissociates in water, there is an increase in hydrogen bonding and the crystalline structure is disrupted, causing birefringence, and the gelatinization process is irreversible. It has been speculated that the breaking of the hydrogen bonds "opens up" the starch granule to improve enzymatic digestion in production animals (Lund and Lorenz, 1984; Zobel, 1988; Hoover, 1995; Haralampu, 2000; Altay and Gunasekaran, 2006; Ratnayake et al., 2006; Lewis et al, 2015a; Massuquetto et al., 2018). During the gelatinization process, the granule's semicrystalline structure is disrupted and birefringence is lost (Donald, 2001). Once the food is cooled, retrogradation occurs. During retrogradation, association takes place with the starch molecules and the hydrogen bonds are tightly oriented (Haralampu, 2000). The percentage of gelatinization is dependent on the starch source (i.e. potato, corn, etc.) due to the different composition of amylose and amylopectin within the starch granule, as well as other factors such as processing temperature, water content, the ratio between starch and water, and botanical source. The common temperature range at which starch is known to gelatinize is between 40 to 120°C (Biliaderis, et al., 1980; Thomas et

al., 1998; Björk et al., 1990; Haralampu, 2000; Weurding et al., 2001; Altay and Gunasekaran, 2006; Ratnayake et al., 2006; Schirmer et al., 2015). It is recommended that the water to starch ratio that is used is 0.3:1 (Lund, 1984).

Thomas et al. (1998) reported that the quality of pelleted animal foods can be improved when carbohydrates are present in the food. During thermal processing, heat and water are added to the system; thus, the starch granule composition becomes sticky and improves the quality of the pellet through agglomeration (Hemmingsen et al., 2008). SG has been observed during extrusion due to the addition of water, pressurized environment, and higher temperatures being utilized when compared to pelleting. High amounts of gelatinization are not observed during pelleting with the water content (%) and temperatures used (Svihus et al., 2004a). Research has shown conflicting results with regard to starch gelatinizing during thermal processing. For instance, research has shown that gelatinization only occurs in small amounts under the standard temperature and time combinations utilized (Hemmingsen et al., 2008). In agreeance with Hemmingsen et al. (2008), Moritz et al. (2002a, 2003) and Abdollahi et al. (2013) observed small amounts of SG occurring during the conditioning and pelleting processes from the friction in the pellet die and traditional temperature parameters set for these processes. In contrast, Thomas and van der Poel (1996) observed that gelatinization does not typically occur during the pelleting process due to a lack of moisture and energy present. It has been speculated that the adhering properties of the pellet during thermal processing has improved food efficiency, pellet quality, and reduced food waste instead of gelatinization (Baird, 1973; Gilpin et al., 2002 and Abdollahi et al., 2011). Although research has been conducted, there is still debate whether SG is the reason to why food efficiency is increasing, or if it is attributed to better pellet quality and pellet durability index **(**Moritz et al., 2001).

The objective of experiment 1 was to evaluate differential scanning calorimetry (**DSC**) as a methodology to assess SG of multi-component food processed with different CT and Hygieniser® RT. Based off past literature, it was hypothesized for this study that the DSC would not be a suitable method to assess SG in multi-component animal food that has been processed with 9 CT and RT combinations. For experiment 2, the objective was to assess SG in ingredient sources used in the formulation of commercial animal food when using 2 DSC temperatures. Based off of past literature and the experiment 1 study, SG will not be able to be assessed in multi-component food, but SG will be able to be assessed in native starch sources (purified corn starch) and ground corn when utilizing 2 DSC temperatures. DSC was chosen as a methodology to use because there has not been much research conducted utilizing this methodology with multi-component animal food (Zhu et al., 2016). For experiment 3, Association of Official Analytical Chemists (**AOAC**) 996.11 was evaluated as a methodology to assess total starch (**TS**) content (%) as the percentage of non-resistant starch present within multi-component animal food processed with 3 different CT and 3 Hygieniser® RT. TS was measured in the multi-component animal food samples conditioned utilizing 3 CT and 3 RT to better understand the gelatinization changes that may be occurring during pelleting (Zhu et al., 2016). AOAC 996.11 is a validated enzymatic-colorimetric methodology that is used in cereal grains. The protocol used from AOAC 996.11/Megazyme was option b – analysis of TS content in samples not containing resistant starch; therefore, the hypothesis for experiment 3 was that the non-resistant starch content will linearly decrease as the CT and RT increases.

Few studies have been conducted to determine the degree of gelatinization and TS content (%) of multi-component animal food when using DSC and AOAC methodologies. Therefore, 3 experiments were conducted to 1) evaluate DSC as an effective method to assess

SG in multi-component animal food when utilizing 3 CT and 3 RT, 2) assess the amount of SG that occurred in multi-component food and common ingredients (purified corn starch and ground corn) used in the formulation of animal food when utilizing 2 DSC temperatures, and 3) assess TS content (%) in multi-component animal food that has been conditioned using 3 CT and 3 RT with the AOAC 996.11/Megazyme (amylose and amyloglucosidase) methodology.

DSC is a thermo-analytical method that measures the temperature difference between the sample and reference pans to determine the change in temperature or enthalpy. DSC can be used to measure the difference in enthalpy between processed (i.e. cooked) and unprocessed (i.e. uncooked) samples to calculate the degree of gelatinization in a sample (Altay and Gunasekaran, 2006; Liu et al., 2013). Enthalpy values must be obtained in order to properly use the SG equation. DSC can also be used to measure phase transitions including glass transition and retrogradation (Liu et al., 2013). Biliaderis et al. (1980) states that the DSC is an efficient method to measure the SG phenomena when using various water-to-starch ratios, to observe SG when conclusion temperatures are above 100 °C and to analyze phase transitions. DSC thermograms are analyzed to obtain the onset, peak and conclusion temperatures as well as the change in enthalpy. DSC was chosen as a method to use because it is not frequently studied for use in multi-component food (Zhu et al., 2016). Traditionally, this method is utilized for analysis of purified extracted starches (i.e. potato starch, corn starch, etc.; Wootton and Bamunuarachchi, 1979). AOAC 996.11 is traditionally used to assess TS (%) in many substrates. DSC is an analytical method that uses thermodynamics to obtain enthalpy values while AOAC 996.11 is a validated wet chemistry method that utilized enzymes to break down the starch granule and spectrophotometry methods are conducted to assess readings of the samples for glucose content.

Enthalpy values are obtained from the plots from the DSC thermograms and inputted into the SG equation. For AOAC 996.11, the TS content of samples is calculated using the TS equation.

MATERIALS AND METHODS

Food Manufacturing.

For experiment 1, 2 and 3, a commercial broiler breeder diet was manufactured at the Charles C. Miller Jr. Poultry Research and Education Center using a steam conditioner (C18INF6.5; California Pellet Mill Co., Crawfordsville, IN), Hygieniser® (C18INF6.5ST; California Pellet Mill Co., Crawfordsville, IN) and pellet mill (PM1112-4; California Pellet Mill Co., Crawfordsville, IN). For experiment 1, the experimental design consisted of a 3 × 3 factorial between temperature in the conditioner (75, 85 and 95°C) and RT (80, 160 and 240 s) in the Hygieniser®, thus totaling 9 treatments. There were 4 sample collection locations in total. First, meal samples were collected from the bucket elevator as the mixer was emptying. Next, conditioned mash samples were collected from the conditioner, but prior to entering the Hygieniser®, which served as the after conditioner (**AC**) samples. Then, samples were collected exiting the Hygieniser®, but prior to entering the pellet mill (**AH**) and after exiting the pellet mill prior to entering the pellet chute to the pellet cooler (**AP**). CT in the conditioner and RT in the Hygieniser® served as the main effects. Three replicate trials were conducted on 3 separate d (n = 198 total samples; 66 samples collected each day; 3 total days; 66 x 3 = 198 samples); 66 samples each run; 3 mixer, 9 AC, 27 AH and 27 AP)).

For experiment 2, a 3 x 2 factorial was used where the main effects were starch source and DSC temperature. The starch sources used for this study were uncooked and cooked purified corn starch, uncooked and cooked ground corn and uncooked meal food and finished pelleted food. The cooked purified corn starch and cooked ground corn samples were thermally processed

utilizing an autoclave at 121 °C for 15 min. These samples were autoclaved to provide similar conditions to food manufacturing including the addition of water vapor and pressure under a specific temperature and time combination. The same commercial corn-soybean meal-based broiler breeder food used in experiment 1 was used for this study. The uncooked meal samples were collected from the bucket elevator as the mixer was emptying and the finished pelleted food samples went through the entire food manufacturing process. The finished pelleted samples were collected after being conditioned at a temperature of 95 °C and retained in the Hygieniser® for 240 s and were the most processed samples.

For experiment 3, the same commercial corn-soybean meal broiler breeder diet was utilized for the AOAC 996.11 analysis of TS content (%). For this analysis, only option b from the AOAC/Megazyme protocol was followed to assess the amount of non-resistant starch within the samples.

Sample Preparation-DSC.

For experiment 1, 2, and 3, a commercial corn-soybean meal-based diet was formulated and manufactured at the Auburn University Charles C. Miller Poultry Research and Education Center. All food samples besides the uncooked and cooked corn starch were ground through a 0.05-mm screen using a Retsch grinder. For experiment 1, 10 mg of the food (as-is basis) and 20 µl of deionized water were weighed into an aluminum hermetically sealed pan to create a 1:2 ratio (wt/wt). Zhu et al. (2016) conducted a study utilizing a multi-component pelleted swine food to assess DSC as a potential methodology to assess SG. Only the multi-component food was selected to use for this study because there have not been many studies conducted evaluating DSC for SG in complete animal food. Therefore, this study was conducted to evaluate DSC as a potential methodology to assess SG in a multi-component broiler breeder food. For experiment

2, either 10 mg of the multi-component animal food on an as-is basis, purified corn starch or ground corn (all samples- cooked and uncooked) were weighed into hermetically sealed pans along with 20 µl of deionized water and allowed to equilibrate overnight in a temperature-controlled laboratory. In both experiment 1 and 2, 1:2 (~ 33.3 % food and 66.6 % deionized water) ratio was used between the sample and deionized water based upon the study conducted by Zhu et al. (2016). Other literature has used this ratio to observe an accurate gelatinization temperature. For instance, Stevens and Elton (1971) utilized a 1:2 ratio (starch/water) and set the DSC temperature to reach a conclusion temperature of 100 °C. They observed that gelatinization occurred between 54 – 73 °C for most starch types. Upon conclusion of the first experiment, it was determined that the DSC is not a suitable method to analyze SG in multi-component animal food; therefore, for the second experiment, it was decided that other starch sources needed to be analyzed to determine how simplistic the samples needed to be (purified vs. complex) in order for the DSC to work to assess SG. CS was the purified source chosen. The ground corn served as an intermediate between the purified corn starch and multi-component food due to having less ingredient inclusions when compared to the animal food but more components when compared to the purified corn starch. Lastly, the multi-component food was chosen to evaluate the use of another DSC temperature to potentially analyze SG in these samples. For both experiment 1 and 2, the aluminum hermetically sealed pans were left in an air-controlled lab overnight to equilibrate. Thermal scans of each sample were obtained from the DSC (TA instruments, Q200; New Castle, DE). For experiment 1, the equilibrated pans were placed into the DSC and heated starting at 10 °C and finishing at 200 °C where the temperature increased by 10° C/min. For experiment 2, the DSC temperature was set to reach either 160 or 200 °C where the temperature increased by 10 °C per minute. In this study, 2 DSC temperatures were also utilized to determine

if SG could be achieved during food manufacturing. Due to DSC not being an appropriate method in the first study, the 2 temperatures were used to see if the DSC could potentially work under these parameters to assess SG in all of the starch sources. For experiment 1, 200 °C was selected as the conclusion temperature because Zhu et al. (2016) utilized the 160 ° C on the DSC to asses SG in the cold mash, hot mash, hot pellet and cold pellet multi-component swine food samples but did not use 200 ° C. When utilizing the DSC, Zhu et al. (2016) observed that the J/g obtained were not theoretically possible at 160 °C; therefore, the 200 °C conclusion temperature was chosen to add more energy to the samples, and to observe if gelatinization could be read by the DSC for the multi-component food samples. In experiment 2, 2 DSC temperatures were used to determine if SG could be achieved during food manufacturing. Due to DSC not being an appropriate method in the first study, the 2 temperatures were utilized to see if the DSC could potentially work under these temperature parameters. Zhu et al. (2016) used a conclusion DSC temperature of 160 °C for their study but observed that this temperature did not allow starch gelatinization to be assessed in the multi-component pelleted swine food. However, this temperature was used for experiment 2 in order to determine if the broiler breeder food would be able to have starch gelatinization assessed. The 200 °C temperature was kept for the second experiment to see if the starch sources altered the presence of SG using DSC. For example, in a study conducted by Shogren (1992), starch gelatinization was assessed for corn starch when 11 – 50 % water was used and a conclusion temperature of 200 °C was set on the DSC. Shogren (1992) observed that gelatinization occurred at ~ 190 – 200 °C when 11-30 % water was present but was able to gelatinize at ~ 70 °C when ≥ 30 % water was present in the sample. This study demonstrates that corn starch can gelatinize at higher DSC temperatures if adequate water is added, therefore, this was a basis to set the temperature for the starch sources used in this study.

The onset (T_o), peak (T_p), and conclusion (T_c) gelatinization temperatures and the enthalpy change (ΔH) were calculated by analyzing the DSC thermograms with the DSC software (Universal Analysis 2000 Software, TA Instruments, Inc.). Based off of previous literature, the DSC thermograms were analyzed between 60 -80 °C to assess gelatinization in all samples.

An equation was utilized to determine the percentage of DG: $\text{DG } \% = (\frac{\Delta H0 - \Delta H1}{\Delta H0})$ where ΔH_0 represents the enthalpy of uncooked samples or native starch (J/g) and ΔH_1 represents the enthalpy of cooked samples (J/g). If a sample is completely cooked, then the DG will be 100 %, whereas if the sample is raw or completely uncooked, then the DG is 0 % (Zhu et al., 2016). All samples were analyzed in triplicate.

Reagent Preparation

The 100 assay Megazyme kit was used which contained 6 bottles: Thermostable α-amylase (bottle 1), amyloglucosidase (bottle 2), GOPOD reagent buffer (bottle 3), GOPOD reagent enzymes (bottle 4), D-glucose standard (bottle 5) and the maize starch control (bottle 6). In addition to the bottles provided in the kit, MOPS buffer (50 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide was prepared as well as sodium acetate buffer (200 mM, pH 4.5) plus sodium azide (0.02 % w/v). The additional reagents were prepared in reference to the Megazyme protocol.

AOAC 996.11/Megazyme Protocol

The AOAC 996.11 Starch (Total) in Cereal Products enzymatic calorimetric method is used to determine the concentration of dietary starch in food by converting the alpha-linked glucose to free glucose using D-glucose controls (McCleary et al., 2019). The objective of this study was to use the AOAC 996.11 approved procedure to determine the amount of starch present in unprocessed and processed samples when 9 CT and RT were used. For each trial, 3

mixer samples were collected for a total of 9 samples (3 trials total; 3 x 3 = 9 total mixer samples). All mixer samples were averaged to determine the percentage of TS within the uncooked meal samples. Samples were also collected at 4 locations at the Charles C. Miller Poultry Research and Education Center. The first location where samples were collected was at the bucket elevator as the mixer was emptying; these samples served as the uncooked meal samples. Next, high moisture mash samples were collected from the conditioner prior to entering the Hygieniser®. Then, high moisture mash samples were collected from the Hygieniser® prior to entering the pellet mill and lastly, the pelleted food was collected leaving the pellet mill prior to entering the pellet chute to the pellet cooler. To determine TS content, an experiment was conducted utilizing the enzymatic colorimetric assay kit and the TS Amyloglucosidase/α-amylase protocol obtained from Megazyme International Ltd. (Wicklow, Ireland, 2011) which is based upon the AOAC International method 996.11 (2007) and AACC method 76.13. With regard to the Megazyme protocol, samples were prepared in accordance to sample b: "Determination of starch in cereal and food products *not* containing resistant starch. D-glucose and/or maltodextrins." Using a Retsch grinder, food samples collected from all locations were ground through a 0.5-mm screen; 100 g of the milled sample were then weighed and placed into borosilicate test tubes. The tubes were then tapped lightly on the counter to make sure that all of the weighed sample was in the bottom of the tube; next, 0.2 mL of 80 % (v/v) aqueous ethanol was added followed by 0.3 mL of thermostable α-amylase was added to the tube. The sample tubes were then vigorously mixed using a vortex mixer. Samples were placed into a 100 °C water bath for a total of 6 mins (samples were stirred at 2, 4, and 6 mins). Tubes were then placed into a 50°C water bath for 10 mins to allow the temperature to equilibrate. Then 4 mL of sodium acetate buffer was added to the tubes followed by 0.1 mL of amyloglucosidase. Next, the

tubes were stirred using a vortex mixer and incubated for 30 mins at 50 °C. The samples contained between 10-100 % starch, so, 7.3 mL of distilled water was added to the test tube to get a total volume of 10 mL. Each of the tubes were weighed and placed into the centrifuge cartridges accordingly to be balanced and the centrifuge was set to run for 10 mins at 3,000 rpm. After removing the tubes from the centrifuge, duplicate aliquots were made containing 0.1 mL of the diluted solution and 3.0 mL of the GOPOD reagent. Two reagent blanks and 4 D-glucose controls were also prepared; the sample tubes, reagent blanks (0.1 mL of distilled water and 3.0 mL of the GOPOD Reagent), and D-glucose controls (0.1 D-glucose standard solution and 3.0 mL of GOPOD Reagent) were incubated at 50 °C for 20 mins. The absorbance values were read for each sample, the D-glucose controls and the reagent blanks at 510 nm utilizing a NanoDrop™ One spectrophotometer (ThermoFisher Scientific, Waltham, MA).

For sample analysis, the Mega-Calc Total Starch (KTSTA) Determination (Solids) calculation worksheet was utilized to obtain the percentage of TS within the samples. This calculation worksheet automatically calculates the as-is basis as numbers are typed into the spreadsheet; however, the equation can be calculated manually; the equation that is utilized to determine total starch content on an as-is basis is (Megazyme; McCleary et al., 1994):

$$\text{Total Starch} = \Delta E \text{ X } F \text{ X } 1000 \text{ X} \frac{1}{1000} \text{X} \frac{100}{W} \text{ X} \frac{162}{180} = \frac{(\Delta E \text{ X } F)}{W} \text{X } 90$$

In this equation, the ΔE represents absorbance once the amyloglucosidase step has been completed and read against the reagent blank, F is a conversion factor from absorbance values to micrograms of glucose, the volume correction value utilized is 1000; the 1/1000 represents a conversion from micrograms to milligrams; W is the sample weight, 100/W is a factor utilized to express the total starch content of a sample as a percentage of the total sample weight and lastly, the 162/180 converts the free glucose left in the sample to anhydroglucose (McCleary et al.,

1994). The AOAC 996.11 method has been validated and has shown to be accurate, repeatable, and reliable (McCleary et al., 1994; McCleary et al., 2019).

Statistical Analysis.

Data for all 3 experiments were analyzed using the GLIMMIX procedure of SAS (V 9.4); means were declared significantly different when $P \leq 0.05$. For experiment 1, CT and RT served as the fixed effects. For experiment 2, starch source and DSC temperature served as the fixed effects. And again, for experiment 3, CT and RT served as the fixed effects.

RESULTS AND DISCUSSION

The equation that is used to calculate DG of samples is $\text{DG } \% = (\frac{\Delta H0 - \Delta H1}{\Delta H0})$ where ΔH_0 represents the enthalpy of uncooked samples or native starch (J/g) and ΔH_1 represents the enthalpy of cooked samples (J/g). This equation was used in both experiment 1 and experiment 2. The DG of multi component food samples was unable to be calculated using the DSC analytical methodology due to the uncooked enthalpy (ΔH_0) being greater than the cooked enthalpy (ΔH_1). The interaction of other non- starch components including proteins, fats and enzymes in the diet may have prohibited accurate analysis of DG utilizing DSC for multi-component food samples. Zhu et al. (2016) also could not accurately measure SG in multi-component food using DSC. In this study, it was observed that overlapping could have occurred from protein denaturation from the corn-soybean meal present in the diet (Zhu et al., 2016). In experiment 1 and 2, ~ 58.4 % corn and 35 % soybean meal were included in the diet which accounts for ~ 37.55 % starch included in the diet formulation. Therefore, it would not be uncanny if interactions were occurring between proteins or other non-starch polysaccharides. Figure 1 displays a DSC thermogram from a finished pelleted food sample set to a conclusion temperature of 160 °C on the DSC. No particular pattern is being followed at this conclusion

temperature. The DSC thermogram for all of the pelleted food samples did not follow a particular pattern and the enthalpy values were too negative/smaller than those for the uncooked samples; therefore, gelatinization could not be analyzed for the multi-component food samples.

In general, the makeup of the starch granule is complex and difficult to study; therefore, the results for SG utilizing a DSC can be inconsistent (Yu and Christie, 2001). Research has demonstrated that the degree of gelatinization is highly dependent on the amount of water present in the starch granule (Svihus et al., 2004b). Upon preparing their starch samples for analysis, Yu and Christie (2001) used three methods. One of the three methods was to weigh the pan and lid to obtain the mass. Next, the starch sample was placed and weighed inside of the pan to obtain the mass of the sample. Next, water was added utilizing a 25 µl syringe. The sample was mixed together using a needle. Although this particular method aids in ensuring that the measurement of the water and starch are accurate, the starch and water mixture may not be homogenous at ≥ 24 h. This may be due to the additional amounts of the water added to the pan. In addition, this may be due to too little water being added to create a mixture; therefore, some inadequate results may be obtained utilizing this methodology. The method used for our study was similar to Yu and Christie. The pan and lid were weighed alone, then the starch sample and lastly the 20 µl was added to the sample using a pipette for accuracy. The samples were allowed to equilibrate overnight, but there is a possibility that all of the water was not absorbed.

In a study conducted by Skoch et al. (1981), maize starch did not gelatinize under CT at 65 and 80°C for 5 s indicating that the moisture content added, and the time limit used was not sufficient for gelatinization to occur. This may be due to the inadequate amount of moisture added during the pelleting process; in addition, the temperatures used may not be high enough. During pelleting, starch has been observed to gelatinize between 5 to 30 %.

Moritz et al. (2001) stated that SG had a linear correlation with the moisture content of the pelleted food; as the amount of moisture added during the pelleting process increased, SG increased. For instance, as the moisture content of the mash food increased from 6.97 to 14.47 %, the gelatinization percentage increased from 6.97 to 13.11 %, respectively (Moritz et al., 2001). The initiation of SG occurs with the addition of heat and water; therefore, this observation may be attributed to the initiation of gelatinization (Moritz et al., 2001).

Donald (2001) observed an inverse relationship between water content and the percentage of gelatinization occurring. As the water content decreased, the gelatinization temperature increased; however, with excess water, most starch types are able to gelatinize at temperatures between 50 to 70°C (Svihus, 2014). Low amounts of gelatinization are obtained during the pelleting process; Svihus et al., (2004b) observed a gelatinization percentage of 19 and 1 % when diets were ground with the hammer mill using a 3 mm screen and 6.1 mm screen, respectively. In addition, when a roller mill was used, gelatinization percentages of 14 %, 5 %, and 17 % were observed with grind sizes at 3 mm with a distance of 0.1 mm, 6.1 mm with a distance of 0.35 mm and a distance of 1.5 mm on the upper rolls and 1 mm on the lower rolls, respectively. Due to limited amounts of water being present in the food during pelleting, the gelatinization temperature could have increased beyond 50 to 70°C which is not achieved during typical pelleting. During the pelleting process, gelatinization has been reported to occur when CT reach approximately 80 °C and when moisture levels were higher than 17 % (California Pellet Mill Co.).

Lewis et al. (2015a) demonstrated that the percentage of gelatinized starch increased as CT and RT increased. The percentage of gelatinized starch was higher at a CT of 88°C for 60 s when compared to a temperature of 77°C for 15 or 30 s (Lewis et al., 2015a).

Gelatinization percentage has been observed to be altered depending on the manufacturing temperatures and times used. Mortiz et al. (2005) observed that gelatinization occurred during the pelleting process from 0 to 28 % when corn was conditioned at 65.6°C for 10 s.

Zhu et al. (2016) used 3 methodologies to determine TS (AOAC 996.11) and DG (DSC and glucoamylase) in a complete pelleted swine food; however, for our study we utilized DSC to determine gelatinization in a complete broiler breeder pelleted food. In both studies by Zhu et al. (2016), it was determined that the DSC is not a suitable method to determine gelatinization due to the interaction from other non- starch matrices. For the study conducted by Zhu et al. (2016), the swine diet was conditioned at 77 or 88°C and retained for 15, 30 or 60 s, opposed to our study which consisted of 3 CT at 75, 85 and 95°C combined with a RT of either 80, 160 or 240 s. Although the time and temperature points exceeded the temperature and time parameters used in the study by Zhu et al. (2016), a logical pattern was not observed with regard to the AH and AP samples at increasing time and temperature points. When returning to the hypothesis for experiment 1, the DSC was not a suitable method to utilize to assess SG in multi-component animal food due to the enthalpy values being higher for the uncooked samples when compared to the cooked. Therefore, the hypothesis for experiment 1 is accepted.

For experiment 1, CT had an effect on the enthalpy of samples. The post-conditioner samples conditioned at 75 °C had higher enthalpy (Table 1.1; $P = 0.0182$) than those conditioned at 85 and 95 °C. Enthalpy of samples collected from the HYG conditioned at 75 °C was higher ($P = 0.0486$) than those conditioned at 85 °C with those at 95 °C being the intermediate (Table 1.1). For samples collected AP, samples conditioned at 75 °C had higher enthalpy than those collected at 85 °C and 95 °C ($P = 0.0025$).

Differences were observed in relation to the effect of RT on AP samples, with samples collected at 80 s having the highest enthalpy, 240 s being the intermediate and 160 s having the lowest enthalpy value (Table 1.2; $P = 0.0445$). The inconsistent results in both the AH and AP processing steps allows us to speculate that there is a change in starch granule composition and SG. The granules can be in different chemical stages along the SG pathway. An interaction (Table 1.3; $P = 0.0200$) was observed between CT and RT for the AP samples. The enthalpy of samples collected AP with the diet conditioned at 75 °C and retained in the Hygieniser® for 80 s have the greatest enthalpy (54.9 J/g) when compared to all other time and temperature combinations. The AP sample at 80 s and 75 °C is the most unprocessed sample whereas the sample at 240 s at 95 °C is the most processed sample. Since the DSC measures how much energy is left in the sample, there should be more energy left in the more unprocessed samples compared to the most processed; however, this is dependent on the step in the transition. This may be attributed to the starch granule organization being structured differently among the SG continuum; in other words, undergoing a different phase change. AOAC 996.11 was conducted following this study to determine the amount of starch in our samples to better understand the composition of the multi-component animal food samples and to later analyze SG with the glucoamylase method.

For experiment 2, enthalpy (H_1) differed among cooked starch sources (Table 2.2; $P = 0.0800$) and DSC temperature ($P = 0.0429$); however, enthalpy did not differ when there was an interaction between starch source and DSC temperature for cooked samples ($P = 0.2166$; Table 2.3); Enthalpy (J/g) did not differ among uncooked starch sources (Table 2.1; $P = 0.1516$); DSC temperature did not affect uncooked samples ($P = 0.6533$; Table 2.2). No significant interaction was observed between starch source and DSC temperature on uncooked enthalpy (J/g; $P =$

0.7683; Table 2.3). Calculated DG of CS was 71.3 %, whereas the DG for GC and finished pelleted multi-component animal food could not be calculated as the uncooked enthalpy (ΔH_0) for both sources was greater than the cooked enthalpy (ΔH_1). Figure 3 shows the CS analyzed for SG at a conclusion temperature of 160 °C on the DSC whereas Figure 5 shows the CS DSC thermogram reaching a conclusion temperature of 200 °C. Both of these DSC thermograms follow a pattern and the SG was able to be calculated for the uncooked and cooked samples when the DSC thermograms were analyzed between 60 – 80 °C (Figure 4). Figure 6 displays the DSC thermogram for the ground corn samples when analyzed to a conclusion temperature of 160 °C and Figure 7 displays the DSC thermogram for the ground corn samples analyzed to a conclusion temperature of 200 °C. Both DSC thermograms were analyzed between 60 to 80 °C. Although these samples were more consistent to analyze than the multi-component food samples at both DSC conclusion temperatures, the SG could not be calculated for these samples due to having negative enthalpy values when compared to the uncooked sample's enthalpy. Ground corn still has some potential interference of non-starch polysaccharides and protein denaturation that could be occurring. Enthalpy values were inconsistent and could not be used to accurately access SG in the multi-component food samples (Figure 1 and Figure 2). Wootton and Bamunuarachchi (1979) observed an enthalpy of 7.6 cal/g for native maize starch. Zhu et al. (2016) obtained an enthalpy of 3.76 – 3.9 J/g for their processed food samples, however, Zhu et al. (2016) stated that this is not theoretically possible. This was attributed to the native starch only being able to obtain 9 J/g. Zhu et al. (2016) stated that the diet utilized for their study contained ~ 28.4 starch; however, the expected enthalpy values from the DSC for this study should have only been able to reach 1.2 – 1.3 J/g. Based on these results, the DSC is not a suitable method to assess DG in ground corn and multi-component pelleted animal food samples.

The combination of proteins, fats, and other carbohydrate fractions do not appear to allow for the use of DSC to measure DG in multi-component animal food. DSC analysis is difficult to utilize to measure phase transitions and the behavior of starch is complex as it can undergo many changes when adequate water and heat are added such as crystallization, gelatinization, and melting. These processes are highly dependent on the addition of sufficient water. There is potential for other non-starch polysaccharides to utilize the water provided for starch (Yu and Christie, 2000; Zhu et al., 2016). Our results are in agreeance with Zhu et al. (2016), who reported that the DSC cannot be used to accurately measure the DG in multi-component food samples. However, it was possible to calculate the DG in the purified CS (71.3 %). This data would suggest that the CS is a suitable substrate for analyses using DSC due to the source being purified without any other components present in the sample to interfere with the enthalpy values. Figure 2 shows a more consistent DSC thermogram with regard to a particular pattern for the multi-component food samples. At 200 °C the food samples followed a particular pattern. All of the analyses for the DSC thermograms were completed between the 60-80 °C temperature point based off of literature. This is the typical range in which starch gelatinizes. The peak at ~ 120 °C is from water. A mini study was conducted to ensure that gelatinization was not occurring as the DSC temperature increased during the testing process (i.e. hotter temperature), The analysis of the water only displayed that the majority of the DSC thermograms have a peak apparent at ~ 120 – 140 °C. However, gelatinization could not be calculated for the GC and multi-component food samples due to having negative/smaller cooked enthalpy values when compared to the uncooked samples when both were inputted into the DG equation.

To truly understand the composition of the multi-component food samples utilized in all 3 experiments, the AOAC 996. 11 TS assay was conducted. To accurately assess the entire

content of the starch granule, the non-resistant starch protocol as well as the resistant starch protocol should be utilized. However, for experiment 3, only the non-resistant starch content was analyzed. The effectiveness of an enzymatic assay with starch granules is highly dependent on if the polysaccharides, amylose and amylopectin, are able to dissociate completely (Karkalas, 1985). Glucose is used as a measurement of starch hydrolysis. It is necessary for the glucose to be able to hydrolyze in order to provide accurate calorimetric analysis (Batey and Ryde, 1982; Karkalas, 1985; Zhu et al., 2016). Due to the nature of the assay, the glucose concentration in the multi-component food samples may be overestimated due to the interaction of non- starch components within the food (Zhu et al., 2016). However, it can be assumed that the purified CS samples and the maize control provided in the Megazyme Kit were not overestimated with regard to the TS content because these samples are purified and should not have interference from other ingredients. The amyloglucosidase is utilized to breakdown the α-1,4 and α-1,6 glycosidic linkages whereas the α-amylase is utilized in this assay to hydrolyze only the α-1,4 linkages (Batey and Ryde, 1982; Zhu et al., 2016). It is important to note that this assay is supposed to be used to determine the percentage of TS in cereal grains opposed to multi-component food. The multi-component food may have not been cooked long enough in the water baths to ensure complete hydrolysis of the enzymes (Zhu et al., 2016).

Karkalas (1985) states that the amount of starch that should be obtained from purified starch samples is approximately 99 %. The two maize samples had a starch content of 99.2 and 98.6, respectively.

Lewis et al. (2015a) observed that the percentage of total starch increased when the conditioner temperature increased ($P = 0.041$), but no significant effects were observed with CT and RT. The total starch numbers that were obtained were 35.2 % and 36.1 %. The samples

collected prior to entering the conditioner and pellet mill had the lowest percentage of TS when compared to the mash that was conditioned and pelleted (Lewis et al., 2015a).

McCleary et al., (1994) utilized the TS method to measure the TS content in various cereal starches. The regular maize starch used in this study contained a TS content ranging from 47.5 to 102.7 %.

For experiment 3, CT had the greatest effect on TS content in samples collected after the conditioner at 95 °C compared to 75 and 85 °C (Table 3.1; $P = 0.0002$). Samples collected after the Hygieniser® contained the greatest amount of starch for samples conditioned at 75 °C compared to 85 and 95 °C (Table 3.1; $P = 0.0001$). For conditioned samples, the average TS content was 5 % at 75°C, 3.8 % at 85°C, and 1.78 % at 95 °C. The average TS content for each CT decreased as the CT increased. For AH samples, TS content ranged between 1-5 %. For AP samples, TS ranged from 0.5-2.5 %. (Table 3.1). This is much lower than the total content observed from other multi- component food samples analyzed in other studies. This may be attributed to the presence of glucose in other samples and the interference of protein fractions from other ingredients due to the complete animal food not being a purified source. Zhu et al. (2016) observed that other non-starch polysaccharides can cause interference, and this would not occur with purified starch sources such as CS and potato starch. For example. Kim and Walker (1992) observed that sugars present in ingredients such as DDGS can potentially delay SG because this ingredient is using up the water available for the SG process to occur. In addition, the diet formulated for other studies can have different inclusions of ingredients to influence TS content when compared to this multi-component animal food. RT influenced samples collected AP (Table 3.2; $P = 0.0152$), but not on samples collected AH ($P \leq 0.9339$; Table 3.2). An

interaction was observed between CT and time for the AP samples at 95°C for 160 s (P = 0.0034; Table 3.3).

CONCLUSION

Starch is necessary to include in the diet to provide energy to animals. Chemical modifications and thermal processing have been shown to alter the composition of starch granules to cause gelatinization; however, it is still unknown if gelatinization is occurring at the temperatures utilized during production animal food manufacturing. DSC does not appear to be suitable to detect the percentage of gelatinization in multi-component food due to the interaction of non- starch polysaccharide matrices and proteins. The TS analysis, AOAC 996.11 may be utilized to measure the amount of starch in multi-component animal food, resistant starch, and various purified cereal grains. Again, due to non- starch interactions, starch may not be able to be detected at accurate levels. Zhu et al. (2016) observed that when utilizing the AOAC 996.11 methodology that the amount of TS may be overestimated due to the amount of glucose in the sample. In this process, the glucose is "freed"; therefore, it may be measuring sugars from other ingredients present within the food such as the non-starch polysaccharides. Currently, the AOAC 996,11 and glucoamylase methodologies are the only 2 methods that have shown to be accurate to assess TS and SG in multi-component animal food. More research needs to be conducted to truly understand if starch gelatinization is occurring during the pelleting methods utilized (i.e. CT and RT). It is important to conduct these analyses to truly understand the percentage of TS present, as well as if SG is occurring with the typical CT and RT utilized while manufacturing multi-component animal foods. This knowledge will also give better insight to metabolism and nutrient utilization in the animal when they are fed diets containing high amounts of starch such as in corn- soybean meal- based diets.

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Table 1.1. Effect of 3 conditioning temperatures on energy retention (Joules/g) of thermally processed samples collected after the conditioner, Hygieniser® and pelleting processing steps, and analyzed utilizing differential scanning calorimetry (DSC)[7].

		Conditioning Temperature,°C				
Thermal Processing Step[1]	Unit[6]	75	85	95	SEM[2]	*P*- value
After conditioner[3]	J/g	71.99[a]	15.27[b]	0.35[b]	16.0	0.0182
After Hygieniser®[4]	J/g	22.73[a]	3.63[b]	9.22[ab]	5.0	0.0486
After pelleting[5]	J/g	25.47[a]	4.08[b]	1.72[b]	5.0	0.0025

[1]Mixer (Uncooked Joules/g); mean = 47.412
[2]SEM = Standard Error of the Mean
[3]After conditioner – Model #: C18INF6.5; California Pellet Mill Co., Crawfordsville, IN
[4]After Hygieniser® - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN
[5]After pelleting – Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN
[6]Energy retention = J/g = Joules/gram
[7]Differential Scanning Calorimetry = TA instruments, Q200; New Castle, DE
[a,b]Means within a row with different superscripts differ at $P \leq 0.05$

Table 1.2. Effect of 3 retention times on energy retention (Joules/g) of thermally processed samples collected after the Hygieniser® and pelleting processing steps and analyzed utilizing differential scanning calorimetry (DSC)[6].

Thermal Processing Step[1]	Unit[5]	Retention Time , s			SEM[2]	*P*- value
		75	85	95		
After Hygieniser®[3]	J/g	17.34	1.00	17.29	5.0	0.0616
After pelleting[4]	J/g	19.72[a]	1.60[b]	10.00[ab]	5.0	0.0445

[1]Mixer (Uncooked J/g); mean = 47.412
[2]SEM = Standard Error of the Mean
[3]After Hygieniser®[3] - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN
[4]After pelleting - Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN
[5]Energy retention = J/g = Joules/gram
[6]Differential Scanning Calorimetry = TA instruments, Q200; New Castle, DE
[a,b]Means within a row with different superscripts differ at $P \leq 0.05$

Table 1.3. Effect of 3 conditioning temperatures and 3 retention times on energy retention (Joules/g) of thermally processed samples collected after the Hygieniser® and pelleting processing steps and analyzed utilizing differential scanning calorimetry (DSC)[6].

	Conditioning Temperature ,°C										
	75			85			95				
	Retention time in the Hygieniser®, s										
Thermal Processing Step[1]	80	160	240	80	160	240	80	160	240	SEM[2]	*P*-value
After Hygieniser®[3], J/g	41.04	0.26	26.91	8.59	1.99	0.30	2.39	0.61	24.67	10	0.109
After pelleting[4], J/g	54.93[a]	4.26[b]	17.21[b]	0.72[b]	0.38[b]	11.14[b]	3.51[b]	0.13[b]	1.52[b]	9	0.020

[1]Mixer (Uncooked J/g); mean = 47.412

[2]SEM = Standard Error of the Mean

[3]After Hygieniser® - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN

[4]After pelleting – Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN

[5]Energy retention = J/g = Joules/gram

[6]Differential Scanning Calorimetry = TA instruments, Q200; New Castle, DE

[a]Means within a row with different superscripts differ at $P \leq 0.05$

Table 2.1. Assessment of starch source on energy retention (Joules/g) of uncooked and cooked purified corn starch, ground corn, and pelleted animal feed utilizing differential scanning calorimetry (DSC).

	Starch Source				
Energy Retention	CS[2]	GC[3]	PAF[4]	SEM[1]	*P*- value
Uncooked, J/g[5]	2.65	1.56	23.24	9.0	0.1516
Cooked, J/g (ΔH1)[7,9]	0.78	73.40	17.73	16.0	0.0800

[1]SEM = Standard Error of the Mean

[2]CS = Corn Starch (purified; Argo)

[3]GC = Ground Corn

[4]PAF = Pelleted Animal Feed

[5]J/g = Joules per gram

[6]ΔH0 = gelatinization enthalpy purified native starch sources

[7]ΔH1 = gelatinization enthalpy of cooked samples

[8]Uncooked = Samples did not undergo thermal processing (autoclaving (corn starch and ground corn) or food manufacturing(pelleted animal food))

[9]Cooked = purified corn starch and ground corn were cooked utilizing an autoclave at 121 °C for 15 min.; pelleted animal food was thermally processed at 95 °C for 240 s

Table 2.2. Assessment of 2 differential scanning calorimetry temperatures on energy retention (Joules/g) of uncooked and cooked purified corn starch, ground corn, and pelleted animal food.

	Differential Scanning Calorimetry Temperature ,°C			
Energy Retention	160	200	SEM[1]	*P*- value
Uncooked, J/g[2] (ΔH0)[3,5]	6.89	11.41	7.0	0.6533
Cooked, J/g (ΔH1)[4,6]	50.07[a]	11.19[b]	13.0	0.0429

[1]SEM = Standard Error of the Mean
[2]J/g = Joules per gram
[3]ΔH0 = gelatinization enthalpy purified native starch sources
[4]ΔH1 = gelatinization enthalpy of cooked samples
[5]Uncooked = Samples did not undergo thermal processing (autoclaving (corn starch and ground corn) or food manufacturing (pelleted animal food)
[6]Cooked = purified corn starch and ground corn were cooked utilizing an autoclave at 121 °C for 15 min.; pelleted animal food was thermally processed at 95 °C for 240 s
[a,b]Means within a row with different superscripts differ at $P \leq 0.05$

Table 2.3. Effect of 3 starch sources and 2 differential scanning calorimetry temperatures on energy retention (Joules/g) of uncooked and cooked purified corn starch, ground corn, and pelleted animal food.

	Starch Source							
	CS[2]		GC[3]		PAF[4]			
	Differential Scanning Calorimetry Temperature ,°C							
Energy Retention	160	200	160	200	160	200	SEM[1]	*P*- value
Uncooked, J/g[5]	3.50	1.85	1.34	1.78	15.90	30.59	12.0	0.7683
Cooked, J/g(ΔH1)[7,9]	1.06	0.50	113.92	32.85	35.23	0.23	23.0	0.2166

[1]SEM = Standard Error of the Mean
[2]CS = Corn Starch (purified; Argo)
[3]GC = Ground Corn
[4]PAF = Pelleted Animal Food
[5]J/g = Joules per gram
[6]ΔH0 = gelatinization enthalpy purified native starch sources
[7]ΔH1 = gelatinization enthalpy of cooked samples
[8]Uncooked = Samples did not undergo thermal processing (autoclaving (corn starch and ground corn) or food
[9]Cooked = purified corn starch and ground corn were cooked utilizing an autoclave at 121 °C for 15 min.; pelleted animal food was thermally processed at 95 °C for 240 s.
Manufacturing (pelleted animal food)
[9]Cooked = purified corn starch and ground corn were cooked utilizing an autoclave at 121 °C for 15 min.; pelleted animal food was thermally processed at 95 °C for 240 s

Table 3.1. Effect of 3 conditioning temperatures on total starch content (%) of multi-component broiler breeder food samples thermally processed after the conditioner, Hygieniser®, and pelleting processing steps.

		Conditioning Temperature ,°C				
Thermal Processing	Unit[6]	75	85	95	SEM[1]	*P*- value
After conditioner[2]	Total Starch, %	5.11[a]	3.81 [a]	ND [5,b]	0.9	0.0002
After Hygieniser®[3]	Total Starch, %	2.35[b]	5.54[a]	1.09[b]	0.6	<0.0001
After pelleting[4]	Total Starch %	1.10	0.51	2.47	0.7	0.111

[1]SEM = Standard Error of the Mean

[2]After conditioner – Model #: C18INF6.5; California Pellet Mill Co., Crawfordsville, IN

[3]After Hygieniser® - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN

[4]After pelleting – Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN

[5]ND = below detectable limit of assay

[6]Total Starch = % of non-resistant starch content detected in the multi-component food utilizing option b for the Megazyme Total Starch Assay Kit

[b]Means within a row with different superscripts differ at $P \leq 0.05$

Table 3.2. Effect of 3 retention times in the Hygieniser® on total starch content (%) of multi-component broiler breeder food thermally processed after the Hygieniser®, and after pelleting processing steps.

		Retention time in the Hygieniser®, s				
Thermal Processing	Unit[5]	80	160	240	SEM[1]	*P*- value
After Hygieniser®[2]	Total Starch, %	3.18	2.96	2.85	0.6	0.9339
After pelleting[3]	Total Starch, %	ND[4,b]	2.41[a]	1.88[a]	0.7	0.0152

[1]SEM = Standard Error of the Mean

[2]After Hygieniser® - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN

[3]After Pelleting – Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN

[4]ND = below detectable limit of assay

[5]Total Starch = % of non-resistant starch content detected in the multi-component food utilizing option b for the Megazyme Total Starch Assay Kit

[a]Means within a row with different superscripts differ at $P \leq 0.05$

Table 3.3. Effect of 3 conditioning temperatures and 3 retention times on total starch content (%) of multi-component broiler breeder food thermally processed after the Hygieniser®, and after pelleting processing steps

		Conditioning Temperature ,°C										
		75			85			95				
		Retention time in the Hygieniser®, s										
Processing Step		80	160	240	80	160	240	80	160	240	SEM[1]	*P* – value
After Hygieniser®[2]	TS, %[5]	3.14	1.74	2.17	5.55	5.36	5.71	0.83	1.77	0.66	1.0	0.8530
After pelleting[3]	TS, %[5]	ND[4,d]	4.53^{ab}	0.17^{cd}	0.76^{cd}	0.70^{cd}	0.09^{cd}	0.01 ^{cd}	2.10^{bc}	5.38^{a}	1.0	0.0034

[1]SEM = Standard Error of the Mean

[2]Hygieniser® - Model #: C18INF6.5ST California Pellet Mill Co., Crawfordsville, IN

[3]After pelleting – Model #: PM1112-4; California Pellet Mill Co., Crawfordsville, IN

[4]ND = below detectable limit of assay

[5]Total Starch (TS) = % of non-resistant starch content detected in the multi-component food utilizing option b for the Megazyme Total Starch Assay Kit

[a,d]Means within a row with different superscripts differ at $P \leq 0.05$

Figure 1. Evaluation of differential scanning calorimetry to assess starch gelatinization in multi-component animal food when setting the differential scanning calorimeter (DSC) to a conclusion temperature of 160 °C. The DSC thermogram shows that there was no consistent pattern when the multi-component animal food sample was assessed for starch gelatinization when utilizing DSC. The peak that is shown would give a negative value.

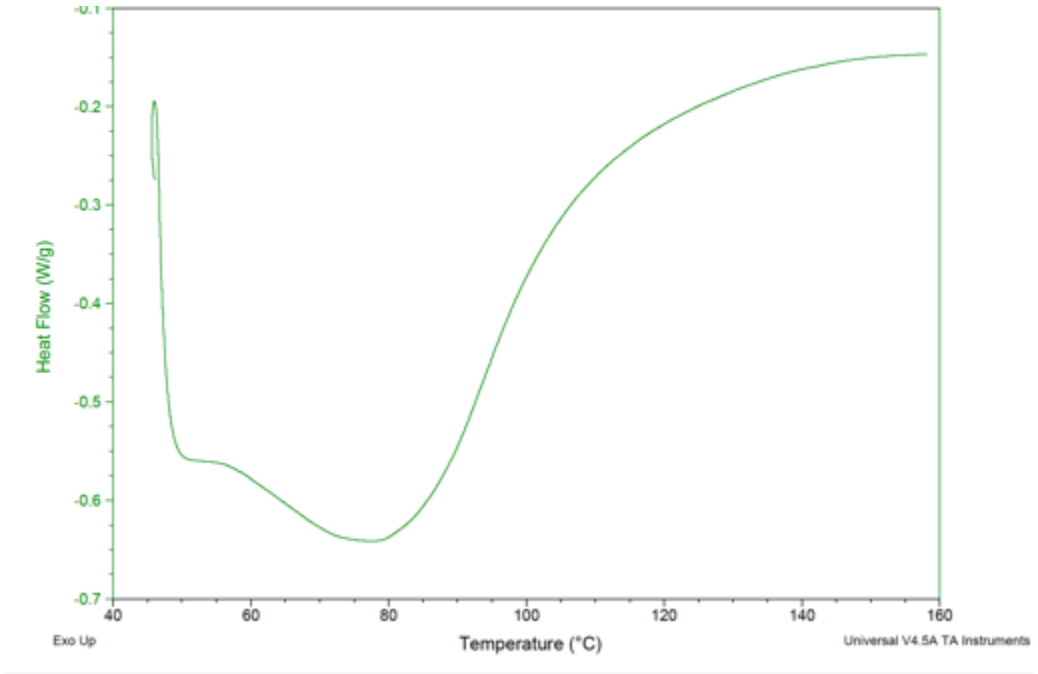

Figure 2. Evaluation of differential scanning calorimetry to assess starch gelatinization in multi-component animal food when setting the differential scanning calorimeter (DSC) to a conclusion temperature of 200 °C. The DSC thermogram shows that there is a distinct pattern for the multi-component animal food samples analyzed with a conclusion temperature of 200 °C, though no gelatinization appears to have occurred between the 60 and 80 °C.

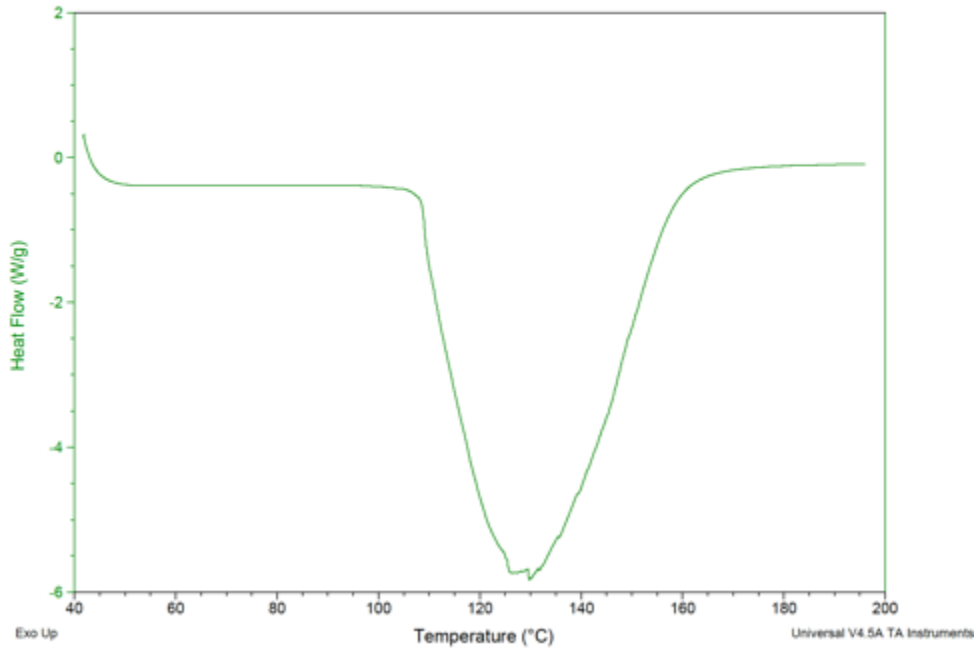

Figure 3. Evaluation of differential scanning calorimetry to assess starch gelatinization in purified corn starch with a differential scanning calorimeter (DSC) conclusion temperature of 160 °C. The DSC thermogram shows that there is a distinct pattern. Starch gelatinization was analyzed between the 60 and 80 °C.

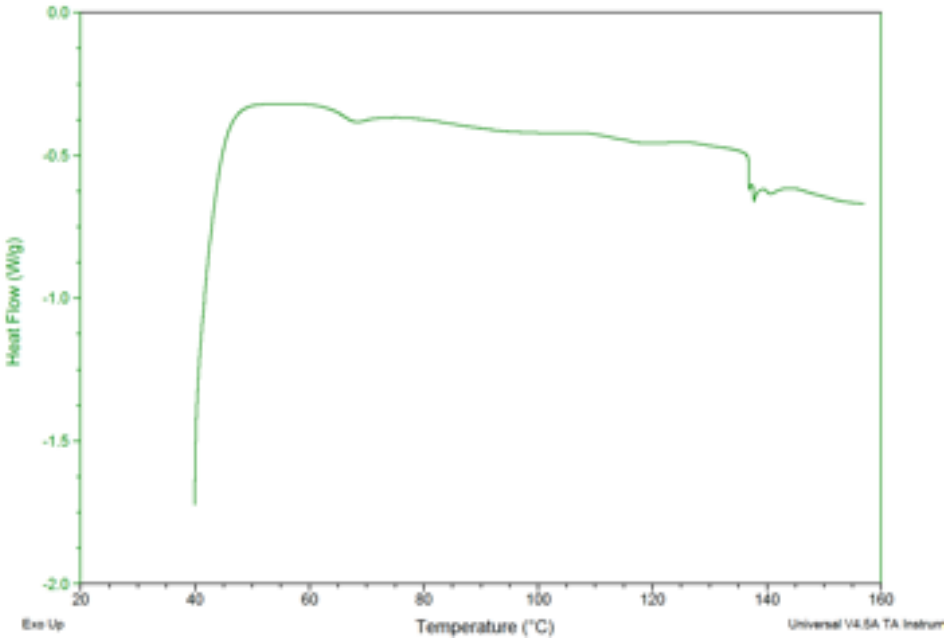

Figure 4. Assessment of starch gelatinization in purified corn starch with a differential scanning calorimeter (DSC) conclusion temperature of 160 °C. The DSC thermogram is an expanded view of the thermogram in Figure 3 and shows that there is a distinct pattern. Starch gelatinization was analyzed between the 60 and 80 °C. The red arrow shows the area of the thermogram used for analysis of starch gelatinization of pure corn starch.

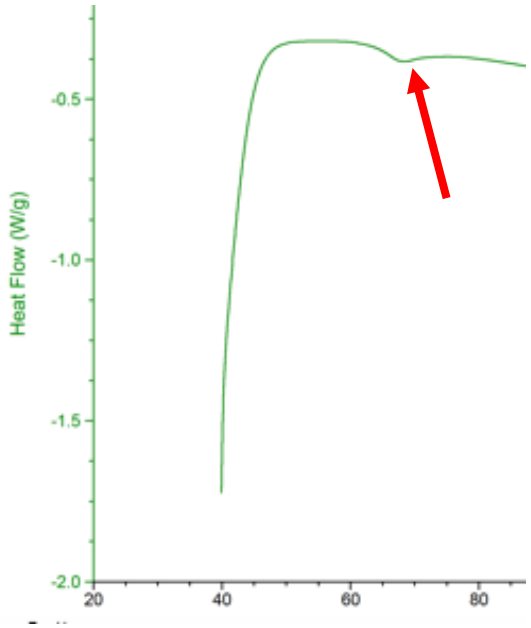

Figure 5. Evaluation of differential scanning calorimetry to assess starch gelatinization in purified corn starch with a differential scanning calorimeter (DSC) conclusion temperature of 200 °C. The DSC thermogram shows that there is a distinct pattern. Starch gelatinization was analyzed between the 60 and 80 °C.

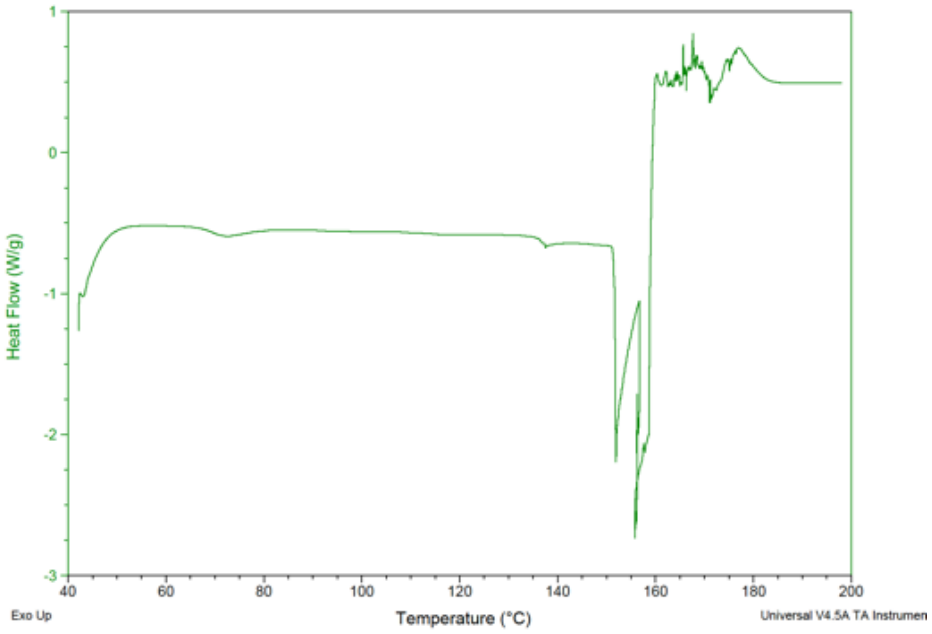

Figure 6. Evaluation of differential scanning calorimetry to assess starch gelatinization in ground corn with a differential scanning calorimeter (DSC) conclusion temperature of 160 °C. The DSC thermogram shows that there is a distinct pattern contrasting with that of the multi-component animal food samples shown in Figure 1. Starch gelatinization does not appear to occur between the 60 and 80 °C and enthalpy values resulting from DSC analysis of ground corn did not allow proper calculation of starch gelatinization of ground corn.

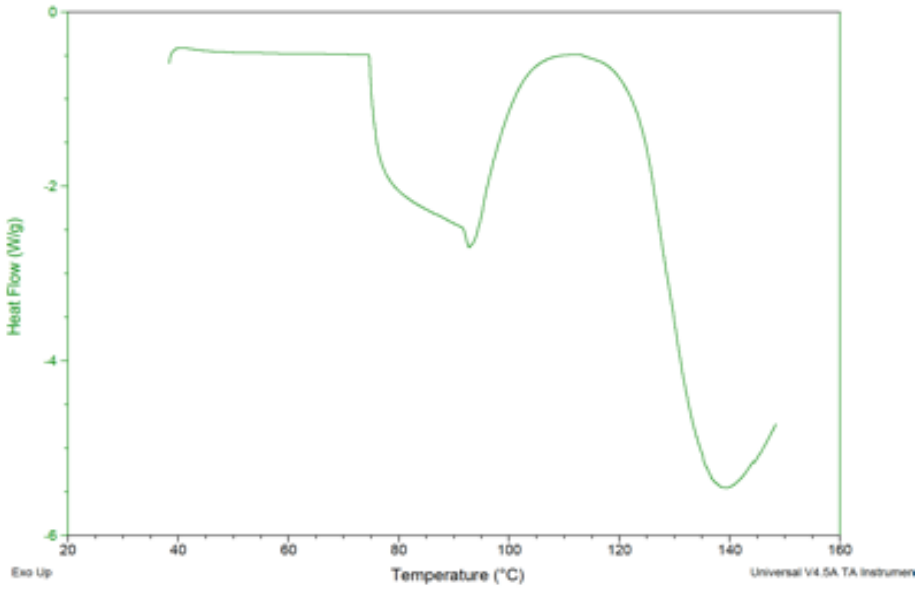

Figure 7. Evaluation of differential scanning calorimetry to assess starch gelatinization in ground corn with a differential scanning calorimeter (DSC) conclusion temperature of 200 °C. The DSC thermogram shows that there is a distinct pattern contrasting with that of the multi-component animal food samples shown in Figure 1. Starch gelatinization does not appear to occur between the 60 and 80 °C and enthalpy values resulting from DSC analysis of ground corn did not allow proper calculation of starch gelatinization of ground corn.