Survey of feed mills around the United States for select bacterial pathogens

by

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Abstract

Feed and feed ingredients are one of the principal sources of microbial contamination in the animal production industry and contamination may occur at any time during growing, harvesting, processing, manufacturing, storage, and distribution. Feed can be a source of *Salmonella*, *Escherichia coli*, *Clostridium perfringens*, and other pathogens of human and animal concern. The objective of this project was to analyze and determine the microbial content of feed ingredients and finished feed (manufactured) from different feed mills around the United States focusing on *Salmonella* spp., *E. coli*, and *Clostridium* spp. Tris phosphate carbonate (**TPC**), which is a pre-enrichment with an strong buffering capacity, and buffer peptone water (**BPW**) were evaluated as pre-enrichment mediums for the recovery of *Salmonella* in feed. A total of 269 samples (feed ingredients and manufactured feed) were collected from 6 feed mills (A, B, C, D, E, and F). Microbial isolation was performed using selective media and colony counts are presented as follows: *Clostridium* spp. counts (**CSC**), *E. coli* counts (**ECC**), *Enterobacteriaceae* counts (**ENC**), aerobic counts (**AEC**), and anaerobic counts (**ANC**). For the pre-enrichment evaluation, pH was measured at 0 and 24 h, samples were plated on selective media to confirm the positive or negative presence of *Salmonella*. Colony forming units, were $\log_{10}$ transformed and analyzed using a GLM model, Proc Glimmix (significant $P \leq 0.05$), and means were separated by sample type using Tukey's HSD. The pH change in time (initial–final) was analyzed using a dependent *t*-test for each sample type in SAS® 9.4 software.

None of the samples were positive for *Salmonella* without pre-enrichment. Differences ($P<0.05$) were observed comparing the feed ingredients with meat and bone meal having the higher CSC counts while DDGS, poultry by-product meal, and soybean meal had lower CSC, ECC, and ENC levels. Additionally, DDGS and poultry by-product meal had lower amounts of AEC and

ANC. Manufactured feed was compared by feed mill. Post mixing samples of feed mill A and E had higher ECC and ENC compared to post cooling and pellet loadout, therefore the pelleting process was effective at reducing the background bacterial contamination in feed ingredients. However, pellet loadout from feed mill E had higher CSC compared to post mixing and post cooling samples which can be presumptive re-contamination and prevalence of *Clostridium* spp. due to it being able to form spores. Manufactured feed was also compared by sample type. Post mixing samples were higher in ECC and ENC compared to post cooling and pellet loadout, however, pellet loadout was higher in CSC than post mixing and mash loadout.

Pre-enrichment evaluation was performed by comparing pH values by sample type for TPC and BPW. DDGS (TPC 6.98/BPW 5.81) and poultry by-product meal (TPC 7.60/ BPW 6.54) presented lower initial pH values for TPC and BPW compared to the other sample types. For both, TPC and BPW, meat and bone meal (TPC 7.77/ BPW 6.45) presented higher final pH values compared to the other sample types, while soybean meal (TPC 5.59/BPW 4.86) and peanut meal (TPC 5.75/BPW 4.68) had lower final pH values. Furthermore, for BPW, post cooling (BPW 5.11), pellet loadout (BPW 5.05) and wheat middlings (BPW 5.15) reported lower final pH values. Most feed ingredients presented significant differences in pH change after 24 h incubation ($P<0.001$) except DDGS. From meat and bone meal samples, 4 *Salmonella* isolates were recovered, 3 using BPW and 1 TPC; for the same samples, initial-final pH for BPW was 6.89-6.40 (*S.* Oranienburg), 6.89-6.45 (*S.* Senftenberg), 7.09-6.42 (*S.* Agona) and for TPC 7.80-7.78 (*S.* Infantis).

Based on these results, the pelleting process is effective in decreasing ECC and ENC, however CSC can persist through this process. Meat and bone meal can be a source of CSC and *Salmonella*. Also, these results show that TPC provided greater buffer capacity compared to BPW, but BPW was more effective at recovering *Salmonella.*

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List of Abbreviations

°C = Degrees Celsius

AEC = Aerobic counts

AFFCO = Association of American Feed Control Officials

ANA = Anaerobic agar

ANC = Anaerobic counts

ASPs = Acid shock proteins

ATR = Acid tolerance response

a_W = Water activity

BAM = Bacteriological analytical manual

BGA = Brilliant Green agar

BHIB = Brain heart infusion broth

BoNT/G = *Clostridium botulinum* neurotoxin type G

BoNTs = *Clostridium botulinum* neurotoxins

BPW = Buffered peptone water pre-enrichment

CDC = Centers for Disease Control and Prevention

cGMPs = Current good manufacturing practices

COA = Certificate of analysis

CP = *Clostridium perfringens*

CPA = *Clostridium perfringens* alpha-toxin

CPB = *Clostridium perfringens* beta-toxin

CPE = *Clostridium perfringens* enterotoxin

CSC = *Clostridium* spp. counts

DAEC = Diffusely adherent *E. coli*

DDGS = Distiller's dried grains with solubles

DNA = Deoxyribonucleic acid

e.g. = exempli gratia (for example)

EAEC = Enteroaggregative *E. coli*

ECC = *E. coli* counts

EFSA = European food safety authority

EHEC = Enterohaemorrhagic *E. coli*

EIEC = Enteroinvasive *E. coli*

ENC = *Enterobacteriaceae* counts

EPEC = Enteropathogenic *E. coli*

et al. = et alia (and others)

ETEC = Enterotoxigenic *E. coli*

ETX = Epsilon toxin

FAO = Food and Agriculture Organization of the United Nations

FDA = Food and Drug Administration

FSMA = Food Safety Modernization Act

g = Gram

GAO = General Accounting Office

GLM = General linear model

h = Hour

HACCP = Hazard analysis critical control point

HCL = Hydrochloric acid

HSD = Honest significant difference

i.e. = id est (in other words)

ISO = International organization for standardization

ITX = Intracellular binary toxin

L = Liter

LB = Lactose broth

$MgCl_2$ = Magnesium chloride

MKTTn = Müller-Kauffmann tetrathionate-novobiocin broth

mL = Milliliter

MLG = Microbiological laboratory guidebook

Na_2CO_3 = Sodium carbonate

Na_2HPO_4 = Disodium phosphate

NaCl = Sodium chloride

$NaHPO_4$ = Sodium phosphate

NE = Necrotic enteritis

NetB = *Clostridium perfringens* necrotic enteritis B-like toxin

NetB = Necrotic enteritis B-like

NTS = Non-typhoidal *Salmonella*

PBS = Phosphate buffered saline

PCA = Plate count agar

PCR = Polymerase chain reaction

pH = Potential of hydrogen

r = Pearson coefficient

RB = Rabbit blood

RV= Rappaport-Vassiliadis

RVS = Rappaport-Vassiliadis with soy broth

SB = Sheep blood

SNE = Subclinical necrotic enteritis

spp. = Species

TPC = Tris phosphate carbonate pre-enrichment

TSA = Tryptic soy agar

TSC = Tryptose sulfite cycloserine agar

TT = Tetrathionate

TTB = Tetrathionate brilliant green broth

UPB = Universal pre-enrichment

US = United States

USDA = United States Department of Agriculture

XLD = Xylose Lysine Deoxycholat agar

XLT4 = Xylose lysine tergitol 4 agar

µL = Microliter

µm = Micrometer

Chapter 1.0 Introduction

During the last 50 years, agriculture in the United States, as well as globally, has been improved and intensified, causing an increase in reliance on a broad range of manufactured feed products as food for animals destinated for human consumption (Crump et al., 2002). Complete feed or finished feed is obtained in feed mills where plant and animal-based feed ingredients are mixed for specific animal species (GAO, 2000). Feed ingredients may acquire bacteria at any time during growing, harvesting, processing, storage, and distribution of the feed, and the persistence of microflora (bacteria) can decrease grain value via nutritional degradation, physical harm, production of harmful toxins to animal health (Maciorowski et al., 2007), or become a carrier of undesired bacteria. Ingredients can be a source for non-endemic *Salmonella* serovars and other enteric bacteria, including pathogenic *Escherichia coli* (Gosling et al., 2021), and *Clostridium perfringens* (Prió et al., 2001). Enteric health and nutrition (feed) are closely related, and the aforementioned bacteria are part of the causes of enteric disorders in poultry (Hafez, 2011).

Furthermore, since the elevated consumption of animal-based food products in the United States, the quality of the resulting food products and the potential human health impact related to animal-based food-production chain, make ingredients used in animal feed highly relevant (Sapkota et al., 2007). This research looks for fresh data on bacterial content emphasizing *Salmonella* spp, *E. coli* and *Clostridium* spp. Further information helps to prevent contamination of finished feed, create mechanisms of control, and identify possible points of contamination or re-contamination. Additionally, tris phosphate carbonate (TPC) and buffered peptone water (BPW) were evaluated as a pre-enrichment method to recover *Salmonella* and buffer capacity using feed ingredients and manufactured finished feed.

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Chapter 2.0 Literature Review

2.1 Animal feed production

In 2020, global animal feed production increased by 1% to 1,187.7 million metric tons according to Alltech's 2021 Global Feed Survey (Alltech, 2021). Regions like Africa, Europe, Middle East, and Oceania reported a decrease in production while Asia-Pacific, Latin America, and North America reported increases despite the world commerce challenges in 2019; it is estimated that nearly 1000 feed mills closed during 2019-2020. By 2020, approximately 44% of the global animal feed production was destined for poultry, 26% for swine, and 22% for cattle (dairy and beef), the rest was aquaculture and pets according to Alltech's (2021) global feed survey. Four basic steps can be described when producing animal food according to the American Feed Industry Association (2022). First, raw materials are received from suppliers where ingredients are tested and analyzed to ensure quality and safety; second, nutritionist create a formula with available ingredients according to the species of interest and its nutritional requirements; third, the feed mill mixes the ingredients to obtain a finished product according to the formula; last, determine and perform the best way to ship the product, for example, product prepared for retail will be bagged and labeled, and product for commercial use will be shipped in bulk. The animal feed industry is a global business of great economic significance which is directly connected to animal feeding operations responsible to fulfill the increasing protein demand of the growing world population (Fink-Gremmels, 2012).

2.1.1 Receiving and storage

It is a fundamental notion that poor quality ingredients result in a poor-quality ration (Jobling et al., 2001). Quality control of ingredients is very important and should be requested by all suppliers and at the same time performed by the feed manufacturer. Quality of ingredients may

vary between shipments or within the same shipment, nevertheless every feed mill should have quality specifications for purchasing different ingredients (ingredient specifications and certificate of analysis), sampling procedures including frequency according to cost of laboratory analysis, supplier reputation and performance (den Hartog, 2003), and be aware of storage space (bins) and time. Stored ingredients for long periods of time, should be analyzed before their use (Cochrane et al., 2016). Prior to storage of new ingredients, the common quality examinations are physical, nutritional, and chemical which includes examination of color, odor, texture, cleanliness, insect infestation, reported moisture, certificate of nutritional content (protein and others), parameters of antinutritional factors (especially in ingredients derived from agricultural products), microbial content, and possible mycotoxin contamination due to high moisture (Tangendjaja, 2015).

2.1.2 Grinding

Grinding is the process used to reduce particle size of cereal grains in animal feed production mills. It is due to the mechanical action of several forces: compression, shearing, crushing, cutting, friction and collision, to which grains are exposed. Using these forces, the mechanical resistance of the grains is exceeded, and their division occurs into several smaller particles of different sizes (Voicu et al., 2013). Reduction of particle size of cereal grains increases the surface area, which consequently improves the interaction with digestive enzymes in the digestive tract of animals increasing nutrient digestibility (Rojas & Stein, 2015; Rojas & Stein, 2016).

2.1.3 Mixing

The process of mixing is one of the most important operations in feed manufacturing and lack of proper mixing can lead to reduced diet uniformity, negatively affecting animal performance (Behnke, 1996) and may cause economic losses. To optimize production, growth, and health,

animals should receive a balanced diet that supplies nutrients and feed additives at proper concentrations (McCoy & Wang, 1994). In the case of mixing, more is not necessarily better, usually there is an optimal mix time according to the specifications of each mixer (Makange et al., 2016).

2.1.4 Pelleting

Feed mills perform several processing operations such as receiving of raw material (feed ingredients), grinding or particle size reduction, proportioning or batching, mixing, conditioning, pelleting, packing, warehousing, and load out and distribution (Abdollahi et al., 2013). One definition of pelleting may be the conversion of finely ground mash feed into dense, free flowing pellets or capsules, in a process that involves steam injection (moisture and heat) and mechanical pressure (Farahat, 2015). The conditioning process improves binding characteristics of feed ingredients and destroys food-borne pathogens (Jones, 2011). The pelleting process involves three activities, conditioning, pelleting, and cooling. The conditioning process is where mash feed is obtained by mixing steam with feed particles, this process involves time, moisture, and temperature. In the pelleting step, continuous compaction of mash takes place through a die with multiple openings and subsequent shearing of the outer portion of the pellet during the extrusion process. Last, the cooling process consists of removing heat by the evaporation of the water and convection of heat from pellets to the cooling air (Thomas & van der Poel, 2020; Abdollahi et al., 2013). According to Munoz et al. (2021), the process of pelleting is very effective at controlling *E. coli* load, decreasing the *E. coli* counts (log_{10}/Gram) from log 3 post-mixer to log 0,08 post cooling.

2.1.5 Contamination

Feed ingredients carry diverse microflora from different environmental sources such as dust, soil, water, or insects. Additionally, feed materials may acquire bacteria at any time during growing, harvesting, processing, storage, and distribution (Maciorowski et al., 2007). According to Maciorowski et al., (2007), the majority of microorganisms developed several strategies to survive until there is sufficient water to support their activity; the persistence of microflora can reduce grain value due to nutritional degradation, physical damage, or production of harmful toxins to animal health. Plant origin feeds can be contaminated in the field where they are grown (i.e. the preharvest stage) by weeds, microbial infection, environmental pollution, or at a later stage like processing at the feed mill (e.g. grinding, mixing, pelleting), storage at the mill, transportation to animal farms, storage at the farms, and dispensing to animals for consumption. Ingredients of animal origin may have the risk of transmitting pathogenic organisms from the farm or processing plant site (rendering) to new animal production farms (Crawshaw, 2012). In the same way, contamination of farm crops and other feeds can occur during transportation or storage where silos, trucks, or other storage areas are poorly cleaned, and the new material is mixed with stored with old material (Crawshaw, 2012).

2.2 Finished feed and contamination

Feed ingredients are important due to their chemical and nutritional components that, together (finished feed), supply the necessary nutrients to the animals for desired performance and development, but can also be a substrate and transmission vectors for unicellular prokaryotic and eukaryotic organisms (Maciorowski et al. 2007). Food-producing animals such as cattle, chickens, pigs, and/or turkeys, are the main causes of many foodborne pathogens like non-Typhi serotypes of *Salmonella enterica*, *Escherichia coli*, and others (e.g. *Campylobacter* species, *Listeria*

monocytogenes) (Heredia & Garcia, 2018). According to Scallan et al., (2011), authors estimated that in the US bacterial enteric pathogens cause 9.4 million cases of foodborne illness in humans, approximately 56,000 hospitalizations, and 1,300 deaths each year; however, only between 1% to 10% of the total cases are reported.

Many factors are involved in the contamination of animal and animal products, including feed, associated fauna, water, animal manure handling, slaughtering, processing practices, and human related animal handling, and is a serious concern because it is hard to control all these factors (Sofos, 2008). Feed mills may implement several common good manufacturing practices (cGMPs) to decrease feed recontamination, for example regularly cleaning feed bins, feed pans, cross augers, hoppers, silos, and transport trucks. Removing caked feed that may be contaminated with pathogenic molds, bacteria, or mycotoxins is also an alternative (Hamilton, 1975; Matuszek & Królczyk, 2017). Disinfection of truck tires while the truck is still moving to decrease soil contamination prior entering to the farm and between the farm and feed mill (Wu et al., 2022). Scrubbing of equipment between shifts has been displayed to decrease attachment of *E. coli* O157:H7 to metal surfaces (Farrel et al., 1998). Sampling of different sources such as feed mixtures, feed ingredients, dust in feed mills to monitor for *Salmonella spp.* (Kwon et al., 2000). Guarantee quality control of feed ingredients and finished feed, including sample monitoring at different points of the mill is a practice that helps to avoid possible contamination during animal feed production.

2.3 General *Clostridium* characteristics

2.3.1 History, natural behavior, structure

The genus *Clostridium* belongs to the *Firmicutes* phylum of bacteria, family *Clostridiaceae,* order *Clostridiales,* and class Clostridia, and is a large heterogenous genus of

microorganisms which includes approximately 231 species; at least 35 are associated with human diseases and 15 produce potent protein toxins (Popoff & Stiles, 2005). According to Boulianne et al. (2020) the *Clostridium* genus is composed of rod-shaped, large, Gram-positive bacteria which are obligate anaerobes and capable to produce spores that allows them to persist in adverse environments like high temperatures and presence of several disinfectants. According to Maczulak (2011), Germany reported several outbreaks of an illness that were linked to eating certain sausages in the late 1700s. Later in 1897, the Belgian biology professor Emile van Ermengem published the finding of an endospore-forming organism isolated from spoiled ham. This isolate was categorized in the genus *Bacillus* by biologists, however, that categorization had difficulties because the isolate grew only in anaerobic conditions while *Bacillus* grew well in oxygen. Genus *Clostridium* and *Bacillus* are similar since both are capable to form a structure called an *endospore*, live primarily in soil, and contain rods and cocci, but the main differences are: *Clostridium* is anaerobe and *Bacillus* aerobe; *Clostridium* form bottle-shaped endospores, and *Bacillus* oblong endospores; and *Clostridium* does not secrete the enzyme catalase, while *Bacillus* have catalase to suppress toxic by-products of oxygen metabolism. Ida A. Bengtson (1881-1952) was the first scientist to assign the van Ermengem's isolate to a new genus called "Clostridium" in her article "Studies on Organisms Concerned as Causative Factors in Botulism" in 1924. Bengtson also contributed by characterizing the tetanus toxin made by *C. tetani* and the botulinum toxin from *C. botulinum. Clostridium* species are widely spread and are found in soils and in the intestinal tract of animals, including humans. Four clostridia species are the main problems for human and animal health, *C. botulinum* (source of lethal toxin and a food-borne pathogen), *C. perfringens* (common cause of foodborne illness and the cause of gas gangrene), *C. difficile* (normal inhabitant of the

intestines that can cause illness), and *C. tetani* (cause of the neurologic disease tetanus) (Maczulak, 2011).

2.3.2 Clostridial diseases

Clostridium species produce at least 18% of the total known bacterial toxins and based on the toxin activity, it can be categorized into three major groups: 1) Toxins that interfere with neurotransmitters, *C. botulinum* causes limberneck and can affect turkeys, chickens, ducks, and mammalian species. 2) Clostridial strains proliferating in the intestines, are the most common and this group is harmful due to its capacity to induce necrotic enteritis (*C. perfringens*), and other intestinal diseases. This group is considered a threat to animal and public health. 3) Clostridia localized in the liver and muscle, capable to produce local or systemic lesions (*C. tetani*) (Boulianne et al., 2020). According to Maczulak (2011), *Clostridium* infection in animals is usually through contaminated wounds or by ingestion of contaminated foods or soil. For instance, C. *botulinum* can cause progressive paralysis which degrades the nerve and muscle over time and finally lethal botulism in animals. *C. botulinum* in birds can be described as "limberneck" due to involuntary movements of the head. C. *perfringens* and C. *difficile* cause gastrointestinal disorders in monogastric, and C. *tetani* bacteria in a wound generate tetanus neurotoxin which is transported through the bloodstream or is absorbed by nerve cells to later reach the spinal column and causes nervous damage. Necrotic enteritis (NE) progresses when C. *perfringens* multiplies chaotically in the intestinal tract of chickens, generating toxins that incentive necrosis (Lovland et al., 2004). According to Skinner et al., (2010), subclinical necrotic enteritis (SNE) is common and hard to diagnosticate, it prevents chickens to reach their average body weight expected by 12% compared to healthy birds and increases the feed conversion ratio by 10%; these factors can be translated in the loss of $0.29/bird.

2.3.3 *Clostridium perfringens* (CP)

CP is a ubiquitous (found everywhere) pathogen that produces several toxins and hydrolytic enzymes; in humans, it can trigger gangrene, gastrointestinal disease, food poisoning and necrotic enteritis, while in animals it is more frequent gastrointestinal and entero-toxemic diseases (Niilo, 1980). This bacterium can produce a large number of toxins that can produce histotoxic, enteric and/or enterotoxemic disease, making it a serious pathogen of humans and animals (Uzal et al., 2014).

C. *perfringens* toxin production is dependent on the strains involved and usually causes lesions of healthy cells (does not invade but harm). A recent categorization of the toxins produced by CP is alpha (α) CPA, beta (β) CPB, epsilon (ε) ETX, iota (ι) ITX, CPE and NetB, and are classified into seven toxino-types (A, B, C, D, E, F, and G), for example CP type F strains produce enterotoxin (CPE) but not CPB, ETX or ITX, and CP type G strains generate necrotic enteritis B-like toxin (NetB) (Rood et al., 2018). CP enterotoxin (CPE) is necessary for human food poisoning (Sarker et al., 1999). CPB is indispensable for the enteric infection of several species by CP (Garcia et al., 2012). The key toxin involved in many CP infections in sheep and goats is ε-toxin (Garcia et al., 2013). CPA is composed of a zinc metalloenzyme constituted by 370 amino acids, is divided into two main domains (catalytic N-domain and membrane binging C-domain) and has a central loop domain containing a ganglioside (GM1a) binding site (Jewel et al., 2015; Oda et al., 2015). Alpha toxin produced by CP induces hemolysis by destroying red blood cells, which results in the failure of the oxygen supply to tissues (Ohtani & Shimizu, 2016). All the CP isolates present the gene encoding CPA which is situated in a stable region within the bacterial chromosome (Uzal et al., 2014). NE in chickens is directly related to NetB (Keyburn et al., 2008) as well as CPA which induces mucosal damage in the intestinal tract of rats, rabbits, and chickens (Miyakawa et al.,

2005). NetB amino acid sequence is similar to the CPB which is a powerful necrotizing lethal toxin (Keyburn et al., 2008). On sheep blood agar, C. *perfringens* produces double zone beta hemolysis (complete brake of red blood cells and hemoglobin) around colonies which is characteristic of this bacterium (Hansen & Elliott, 1980; Miah et al., 2011; Shanmugasamy & Rajeswar, 2012; Dar et al., 2017).

2.3.4 *Clostridium argentinense*

C. argentinense, due to its capacity to produce a botulinal-like toxin, was initially thought to be a variant of *Clostridium botulinum* (Gimenez & Ciccarelli, 1970). It was first discovered and isolated by Gimenez & Ciccarelli (1968) from a soil sample of a cornfield taken in Mendonza, Argentina. Later, it was differentiated from *C. botulinum* because of its inability to metabolize glucose and produce volatile fatty acids, additionally, it is negative for lipase reaction in peptone-yeast extra-glucose broth cultures (Ciccarelli et al., 1977). This organism can be described as a motile, peritrichous, anaerobic, gram-positive rod that produces spores and can produce beta-hemolytic colonies on rabbit blood agar while this capacity is weak or absent on sheep blood agar (Gimenez & Ceccarelli, 1970; Suen et al., 1988). Not all strains of *C. argentinense* can produce neuroparalytic toxins according to Suen et al., (1988), triggering variable data about it in laboratory animal analysis.

To understand more about *C. argentinense* is necessary to talk about *C. botulinum* and botulinum neurotoxins (BoNTs). BoNTs are the most potent naturally occurring substances, only 50 ng of the neurotoxin is enough to cause human botulism (Peck et al., 2017). *C. botulinum* is different from other clostridia due to the production of a neurotoxin that causes botulism paralysis (Suen et al., 1988). Different strains of *C. botulinum* produce seven immunologically distinct botulinal toxins which are classified as A, B, C, D, E, F, and G (Austin, 2003), however, nowadays

at least six physiologically and genetically distinct bacteria to *C. botulinum* are known to produce botulinum neurotoxins as well (Sebaihia et al., 2007; Hill & Smith, 2012). *C. botulinum* different bacteria is categorized into four (I, II, III, IV) groups, group I and II are mostly related to human botulism, group III is accountable for botulism in different animal species, and group IV seems not to have an impact with botulism in humans or animals (Peck et al., 2011; Johnson, 2019). *C. argentinense* was recognized as *C. botulinum* type G (Suen et al., 1988), these strains able to form BoNT/G were assigned to group IV of *C. botulinum* (Sonnabend et al., 1981).

Clostridium argentinense sp. was described by Gimenez & Ciccarelli (1970) as a motile, peritrichous, anaerobic, gram-positive rod, with or without spores. Suen et al., (1988), stated that on rabbit blood the colonies are β-hemolytic, while on sheep blood hemolysis is weak or absent; the bacteria are proteolytic (break down proteins) and gelatinolytic (degrade gelatin), producing acetic, isobutyric, butyric, isovaleric, and phenylacetic acids in peptone-yeast extract-glucose medium. Also, it does not ferment sugars, it is negative in tests for hydrolysis of starch and esculin, and strains may or may not produce a neuroparalytic toxin that is responsible for botulism. There is no documented association of this bacterium or toxin with botulism in humans. Type/G-forming organisms have a relatively low toxigenicity, generating small quantities of toxin in culture (Terilli et al., 2011) making it difficult to differentiate in presence of other species (Sonnabend et al., 1987). It was suggested that BoNT/G genes were on the plasmid of *C. botulinum* type G since the production of BoNT/G stopped when the samples tested lost an 81-MDa plasmid during stressful growth at 44 °C (Eklund et al., 1988; Eklund et al., 1989). Zhou et al., (1995) presented evidence that the genes for three proteins of the toxin complex BoNT/G are clustered on a large plasmid. They state that the plasmid DNA but not the chromosomal DNA of the toxigenic strains tested, served as a template for PCR to amplify an oligonucleotide as the L chain of the BoNT/G, therefore

the genes encoding nontoxic proteins of the type G toxin complex occur as a cluster on the plasmid. Plasmids are small circular DNA molecules that have small numbers of genes and are key players for mobile genetic elements that favors bacterial adaptability and diversity (Thomas, 2000). A problem with plasmids is that replicate independently from the chromosome of their host, and their evolutionary interest may vary from those of the chromosome (Tazzyman & Bonhoeffer, 2014). Variations and some challenges are reported during plasmid DNA isolation mostly with lysing of cells, time of incubation for growing cells, and plasmid formation during stressful growth (may be present, or not) (Strom et al., 1984; Weickert et al., 1986; Eklund et al., 1989). Munoz et al., (2021), reported identification by 16S sequencing of C. *argentinense* in manufactured feed samples from different feed mills (post mixing, post cooling, post pelleting, and loadout) in United States.

2.4 General *Salmonella* and *E. coli* characteristics

2.4.1 *Salmonella*

Salmonella belongs to the family *Enterobacteriaceae* and is a gram-negative, facultative, rod-shaped bacterium. *Salmonella* is extensively spread in nature and causes several diseases in animals (including humans). In animals, salmonellosis is found in four major forms: enteritis, septicemia, abortion, and asymptomatic carriage, while in humans, includes numerous syndromes like enteric fever, gastroenteritis, septicemia, focal infections, and asymptomatic carrier state with typhoidal strains (Agbaje et al., 2011). *Salmonella* foodborne outbreaks occur all over the world and cause important economic losses (Lillehoj & Okamura, 2003). *Salmonella* serovars are identified through their somatic (O), flagellar (H), and capsular (Vi) antigens, more than 2,500 serovars have been identified and have shown a wide host range mainly in warm-blooded animals (including rodents and wild birds) but also some reptiles (Hendriksen et al., 2009; Dunkley et al., 2009). According to their host adaptability, *Salmonella* serovars can be classified into three groups:

1) Adapted to primates, e.g. *Salmonella* Typhi, *Salmonella* Paratyphi A, B, C and *Salmonella* Sendai; 2) Adapted to specific animals, e.g. *Salmonella* Dublin in cattle, *Salmonella* Gallinarum and Pullorum in poultry, *Salmonella* Abortusequi in horses, *Salmonella* Abortusovis in sheep and *Salmonella* Choleraesuis in pigs; 3) Other *Salmonella* species that produce infection in a wide range of animals (including humans), e.g. *Salmonella* Typhimurium and *S.* Enteritidis. These bacteria can survive in diverse environmental conditions, for example *Salmonella* normally grows in temperatures below 54°C but can survive in cold conditions below 2 – 4 °C (Li et al., 2012). Infection by *Salmonella* occurs in the small intestine where the bacteria attach to cells, later the enterocytes are attacked, and the infected epithelial cells rupture into the intestinal lumen resulting in loss of absorbing capability of the intestinal tract (Meerburg & Kijlstra, 2007). *Salmonella* can be spread through feces and animals like rodents, wild birds, and cats can be a source of cross-contamination of *Salmonella* (Davies & Wray, 1997). Following the mentioned route of contamination (fecal-oral), chickens would eat rodent or wild bird feces present in the feed or bedding and get contaminated becoming a vector by secreting or shedding the organism (Meerburg & Kijlstra, 2007). *Salmonella* can be also present in the air, litter, dust, and all equipment in contact with feed ingredients and manufactured feed in the production facilities (Maciorowski et al., 2004). Moisture content of the substrate is a determinant for *Salmonella* survival, it can survive in animal and human foods containing low water activity (a_W) for long periods (Farakos et al., 2013). Williams & Benson (1978) tested *Salmonella* survival ability using poultry feed and litter contaminated with a large number of *S.* Typhimurium, they reported persistence of at least 18 months at 11°C in both feed and litter, while persistence of 16 months in feed and 18 months in litter at 25 °C; at 38 °C persistence of approximately 40 days in feed and 13 in litter, highlighting that feed and litter samples for the bacteriologic examination should be stored at low temperatures.

Additionally, *Salmonella* prevalence in water that is present inside conveyors, storage bins (in the feed mill or poultry houses), leaky roofs, and steam valves (Maciorowski et al., 2004). Jones (2011) states that lack of cleaning practices in the mentioned areas can lead to the formation of biofilms and make *Salmonella* difficult to eliminate.

Feed ingredients can be transmitters of *Salmonella* to manufactured feed and subsequently to live poultry, and products like eggs and meat would be potential sources of these bacteria (Cox et al., 1986; Dunkley et al., 2009). Himathongkham et al. (1996) reported that infection in young chicks (1 - 4 days) can be caused by one *Salmonella* cell, nevertheless, infection in chicks of 6 to 7 weeks of age require various thousand *Salmonella* cells. Sources of *Salmonella* include soil, water, animal feces and several foods such as raw meat (cattle and poultry), eggs, milk, and dairy products (Jones, 2011). The Centers for Disease Control and Prevention (CDC, 2021) estimates *Salmonella* bacteria cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States annually. According to the Food and Drug Administration (FDA, 2013), compliance policy guide sec. 690.800 *Salmonella* in food for animals, under section 402(a)(1) of the FD&C Act (21 U.S.C. 342 (a)(1)), considers pet food adulterated when is contaminated with any *Salmonella* serotype and no commercial heat steps or processes to kill *Salmonella* are applied. Regulatory action over pet food is the same as human food since pets and pet food are in direct contact with humans becoming a potential health risk, zero-tolerance policy for any *Salmonella* contamination under the Food Safety Modernization ACT (FSMA) (FDA, 2011). However, in the case of animal feed (intended for the consumption of animals other than pets) contaminated with *Salmonella*, regulatory action is necessary when the *Salmonella* serotype is known to cause disease in the animal species that will consume the feed, for example poultry feed with *Salmonella* Pullorum, *S.* Gallinarum, or *S.* Enteritidis.

2.4.2 *E. coli*

Escherichia coli is one of the most well-studied bacteria, is a rod-shaped, gram-negative bacteria belonging to the family *Enterobacteriaceae*; its growth and propagation is very fast under optimal conditions (Jang et al., 2017). *E. coli* is commonly present in the vertebrate intestinal tract and is a frequent microbial contaminant of retail meat products (Davis et al., 2018). Most *E. coli* strains are non-pathogenic and stay within the gastrointestinal tract causing no problems, some strains, under proper conditions, have the potential to cause harm to the gastrointestinal tract and cause diarrhea, urinary tract infections and sepsis/meningitis (Kaper et al., 2004). *Escherichia coli* O157:H7 is one of the most important agents responsible for severe gastrointestinal diseases in humans, causing most outbreaks of bloody diarrhea in United States and most cases of hemolytic-uremic syndrome (Boyce et al., 1995; Dean-Nystrom et al., 2003; Silagyi et al., 2009). *E. coli* can be categorized in six well described categories according to their virulence attributes that cause disease in healthy individuals, the categories are: enteropathogenic (EPEC), enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) (Kaper et al., 2004; Nataro & Kaper, 1998). Additionally, there are a group of *E. coli* that are responsible for extra-intestinal disease in chickens, turkeys, and other avian species, this group is avian pathogenic *Escherichia coli* (APEC) (Ghunaim et al., 2014). Despite APEC being responsible of several extraintestinal diseases in poultry, the most economically important diseases for the industry are colibacillosis, airsacculitis/colisepticemia and cellulitis in broiler chickens, and salpingitis/peritonitis in commercial layer chickens (Nolan et al., 2013). APEC is also considered a key source of antimicrobial resistance spreading to other bacteria, mostly by their plasmids and exchange of genetic material (Gyles, 2008). The specific control of APEC is complicated since *E. coli* is part of the normal microflora of the avian gut and

the colonization is basically in the first hours of hatching (Dho-Moulin & Fairbrother, 1999). According to Munoz et al., (2021), ground corn meal and peanut meal may contain higher *E. coli* levels compared to soybean meal, DDGS, corn gluten meal, ground wheat and poultry by-product meal, but diets with these feed ingredients after conditioning and pelleting, showed that the *E. coli* levels dropped in finished feed.

2.5 Methods of control of bacteria in animal feed

Microbial contamination of animal feed is a problem because finished feed and feed ingredients are potential vectors of pathogenic bacteria, and contaminated ingredients can contaminate facility equipment, resulting in cross-contamination of other products (Huss et al., 2015). During monitoring for bacterial contamination, guaranteeing that feed samples are pathogen-free is difficult since the distribution of contaminants is not uniformly dispersed in feed and isolation or detection of organisms (bacteria) may be hindered if they are damaged or injured (Wesche et al., 2009).

Control plans: The first step for pathogen (bacteria) control in animal feed and feed ingredients would be the implementation of a microbial sampling plan and Hazard Analysis Critical Control Point (HACCP) programs setting certain levels of permissible contamination that are not a risk for animal and human health (Crump et al., 2002). According to Butcher & Miles (1995), the identification of critical control points and conducting of hazard analysis should be implemented to reduce possible pathogens entering the fed mill which can potentially affect animals and consumers. All raw ingredients should be inspected looking for rodent contamination, bird droppings or insect infestation, and providers should present a Certificate of Analysis (COA) to ensure quality and biosecurity, subsequently rating the suppliers according to their quality reliability (Phillips et al., 2013; Crawshaw, 2012). Implementation of routine inspections and

sanitization of transportation vehicles should be part of the program, also classification of the vehicles according to what they transport (Butcher & Miles, 1995). Additionally, training of the personnel should be implemented on handling techniques, aseptic sampling, and current good manufacturing practices (cGMPs) to get reliable results following the guidelines with the objective of reducing contamination (Huss et al., 2015). According to Jones (2011), aseptic sampling is required to obtain correct results about contamination rates.

Feed manufacturing: Contamination can also occur during feed manufacturing (cross-contamination), before or after mixing in the case of mash diets and after cooling in the case of pellet diets. Some sources of re-contamination are unclean receiving and unloading areas, unclean intake pits, dust generated by feed ingredients, dirty conveyers, inadequate feed storage conditions, and presence external people in unsanitary clothes (Jones, 2002; Jones & Richardson, 2004). Several pathogens have been found in contaminated animal feed, such as *Salmonella spp*., *Escherichia coli* O157:H7 (Cox et al., 1983; Davies & Wray, 1997; Davis et al., 2003), pathogenic *Escherichia coli* (Gosling et al., 2021), and *C. perfringens* (Prió et al., 2001).

Heat process: During the pelleting process, steam conditioning has been shown to eliminate most contaminating bacteria in feed (Furuta et al., 1980), however, this process is not 100% effective. Jones et al. (1991) reported 82% reduction of *Salmonella* spp. in contaminated feed after pelleting. The efficacy of the conditioning and pelleting process to reduce bacteria is dependent on temperature, retention time, and the microorganism (Jones & Richardson, 2004). *C. perfringens* in manufactured feed is usually detected in its spore form, which is resistant conditioning and pelleting and to chemical preservatives (Sarker et al., 2000).

Transportation, biosecurity, and pest control: Other considerations for bacterial control are the transportation of feed to farms, storage, and to prevent contact of feed with wild or

infected animals that may shed pathogens (Alali & Ricke, 2012). During transportation avoid unclean transportation containers and sudden changes of temperature and humidity. At the farm, regular cleaning of storage bins located outside poultry houses, avoid commingling new feed over old feed, and maintain feed dry to prevent the growth of contaminants like *Salmonella* and *E. coli*. Additionally, implement biosecurity actions like records of who is entering and leaving the facility, regular disinfection of boots/shoes, use of pans with disinfectant (to prevent cross-contamination among areas), cleaning of clothes used in the farm (for all the personnel), and regular control of rodents and wild birds that might be in contact with the feed, equipment, personnel or animals (Meerburg & Kijlstra, 2007; United States Agricultural Research Service: Animal Health Division, 1966).

Additives: Implementation of a good HACCP plan at a feed mill, monitoring, testing and analysis of critical control points are very important procedures to minimize contamination and recontamination of feed (Johnson & Parkes, 2001). Complementary to the preventive procedures, feed additives to control pathogens in the feed are applied since raw ingredients may be contaminated (Alali & Ricke, 2012), these include organic acids and formaldehyde (Ha et al., 1998; Lesson & Marcotte, 1993; Ricke, 2003; Maciorowski, 2004). Organic acids effectiveness can be compromised by many factors such as level of contamination, physical form of the feed, moisture content, quantity of addition, and diet formulation (Jones, 2011; Koyuncu et al., 2013). Other disadvantages using organic acids can be corrosion of the feed mill equipment and compromise of palatability (Jones, 2011).

If the prevention methods to control bacteria in feed do not work, antibiotics are applied. Antibiotics are largely used (without restrictions in many countries) for three main purposes in animals: therapeutic treatment of sick animals, prophylactic use to prevent possible infections

(usually in farms with low biosecurity) and as growth promoters to improve production at low inclusions in animal feed (Barton, 2000). However, many countries have implemented regulatory controls, as a result, many antibiotics are banned for regular use (Mathew et al., 2007).

2.6 Importance of pre-enrichment media for isolation of *Salmonella spp.*

Detecting microorganisms from specific sources can be difficult and proper consideration must be given to the influence of environmental factors before detection (Bissonnette et al., 1975). The exposure to freezing, heating, or freeze-dying negatively impacts detection and enumeration of some microorganisms because of physiologically weakened or injured cells (Clark & Ordal, 1969; Ray et al., 1971; Ray & Speck, 1973). The type of sample, level of stress or injury applied by the sample matrix or environment, presence of commensal bacteria, and level of *Salmonella* in the sample matrix are important considerations for the successful recovery of this bacteria (Busse, 1995).

Poultry feed can be a potential source of *Salmonella* (Cox et al., 1983; Quinn et al., 1995; Veldman et al., 1995; Davies & Wray, 1996; Heyndrickx, et al., 2002). However, a low percentage of samples tested are reported as positive for *Salmonella* (Heyndrickx et al., 2002; Veldman et al., 1995). Standard cultural procedures for the isolation of *Salmonella* generally included pre-enrichment of samples in a non-selective broth medium, enrichment in a selective broth medium, isolation by presumptive screening and serological confirmation of presumptive isolates (D'Aoust, 1981). The use of selective media may hinder the detection of such stressed or injured microorganisms since injured cells become sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies (Bissonnette et al., 1975).

2.6.1 Pre-enrichment, general background

By the 1950s, there were several reports of a two-stage pre-enrichment, enrichment process (Childs & Allen, 1953; Byrne et al., 1955; Walker, 1957). According to Thatcher & Clark (1968), the practice of using pre-enrichment was only recommended when the test material had suffered heating, drying, irradiation, extended freezing or had a low pH. Corry et al. (1969), indicated that multiplication of heat or radiation injured *Salmonella* cells in tetrathionate and selenite broth growth is not recommended except using a pre-enrichment step before. As a result of this, a comparative trial on the isolation of sub-lethally injured *Salmonella* in nine European laboratories was developed with higher isolation rates in the samples processed with pre-enrichment (Edel & Kampelmacher, 1973).

2.6.2 Culture methods to isolate *Salmonella*

Through the years, various comprehensive culture-based methods to detect *Salmonella* in foods were developed due to the impact of this bacterium (food contamination) on public health. The principal and preferred laboratory procedures for microbiological analyses of foods are found explicitly in the Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM), the United States Department of Agriculture (USDA) Microbiological Laboratory Guidebook (MLG), and the ISO 6579:2002 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* species (USDA, 2022; FDA, 2015; ISO, 2002).

The present method used by the FDA, described in the BAM for animal food (dry cat food, dry dog food, cattle feed, horse feed, poultry feed, and swine feed), requires a 24-h recovery in non-selective pre-enrichment broth, preferably buffered peptone water (BPW) or lactose broth (LB), followed by 24-h selective enrichments incubation in Tetrathionate (TT) and Rappaport-Vassiliadis (RV) broths to decrease the growth of competitive bacteria (FDA, 2015). The method

for detection of *Salmonella* described in the International Organization for Standardization (ISO) 6579:2002 consists of non-selective pre-enrichment in BPW, selective enrichment in Rappaport-Vassiliadis with soy broth (RVS) and Müller-Kauffmann tetrathionate-novobiocin broth (MKTTn), plating on selective solid medium Xylose Lysine Deoxycholat agar (XLD) and a second selective solid medium such as Brilliant Green agar (BGA), finally serological and biochemical confirmation (ISO, 2003).

2.6.3 Pre-enrichment, principles, and components

A pre-enrichment medium should not only be a noninhibitor for *Salmonella* but should also be capable of supporting the proliferation of small numbers of these bacteria especially in higher dilutions where any possible nutritional or inhibitory substances from the food being tested should have been diluted (North, 1961). Taking as an example BPW, according to the HIMEDIA® technical data sheet (HiMedia Laboratories, 2022), BPW is a pre-enrichment medium intended to support recovery of sub-lethally harmed *Salmonella* prior to transfer to a selective medium. This medium presents no inhibitors, is well buffered, and offers conditions for resuscitation of food preservation processes injured cells. BPW proposed composition described in the BAM is 1 L distilled water, 10 g of peptone, 5 g of sodium chloride, 3.5 g of disodium phosphate and 1.5 g of mono-potassium phosphate (FDA, 2015). Peptone water provides nitrogenous and carbonaceous compounds, long chain amino acids, and vitamins (essential nutrients). Sodium chloride maintains the osmotic balance of the medium. Disodium phosphate and mono-potassium phosphate are soluble in water and provide high buffer capacity which prevents sudden drops in the pH of the solution (HiMedia Laboratories, 2022). The goal of using pre-enrichment in a non-selective broth medium is to provide for the non-inhibited growth of local bacterial flora and the resuscitation and proliferation of stressed or injured Salmonellae to detectable levels (Litchfield & Insalata, 1973).

Richardson et al. (2019) reported that the pH impact on *Salmonella* was dependent on the serotype and the stress status of the microorganism (liquid sample or dry sample). They reported that pH 4.85 was enough to kill 50% of *Salmonella* Typhimurium in a non-stressed or liquid state during 24 h incubation, while in a stressed state, a pH of 5.85 was enough to kill 50% of the cells. Therefore, the serotype of *Salmonella* recovered can be influenced by the ability of pre-enrichment medium to buffer acidity. It has also been reported that low pH alters the biochemical pathways of *Salmonella* (Blankenship, 1981; Richardson et al., 2019). Richardson et al. (2019) also observed that when *Salmonella* was exposed to pH ranging from 4 to 7 on xylose lysine tergitol 4 agar, it can lose its ability to produce hydrogen sulfide and this effect was dependent on the pH, stress status, and serotype.

Is important to mention that different researchers have reported that the detection of *Salmonella* in feed during pre-enrichment or enrichment can be complicated by the level of background microflora in the sample; the higher the level of background microflora, the lower the recovery of *Salmonella* (Koyuncu & Haggblom, 2009; Kuijpers & Mooijman, 2012; Mooijman, 2018).

Production of animal feed in feed mills is usually in large quantities as bulk material in a batch wise production; *Salmonella* cells numbers in feed are usually low and poorly distributed (Koyuncu & Haggblom, 2009). Maciorowski et al. (2006) mentioned that it is crucial to provide injured and stressed *Salmonella* cells an opportunity to recover and multiply in the pre-enrichment and enrichment before its isolation from animal feed.

2.6.4 Enrichment, principles

The use of Tetrathionate (TT) and Rappaport-Vassiliadis (RV) selective broths is common to all finding methods employed for the recuperation of *Salmonella* in any case of the type of food

or non-selective pre-enrichment broth utilized (Daquigan et al., 2016). Selective enrichment prevents the proliferation of non-salmonellae and simplifies isolation of *Salmonella* on selective plating media (Fagerberg & Avens, 1976).

Tetrathionate broth is a selective medium whose action includes bile salts and brilliant green dye (to inhibit gram-positive bacteria), an iodine-Potassium iodine (I_2-KI) solution (which will induce TT production and provide a metabolic advantage to organisms that have TT reductase like *Salmonella*), and calcium carbonate to neutralize the sulfuric acid produced during TT reduction (thus preventing a sudden drop of pH) (Teague & Clurman, 1916; Knox, 1945; Palumbo & Alford, 1970; Moats et al., 1974; D'Aoust, 1981; Moats, 1981). The ability of *Salmonella* to reduce TT enables its growth in TT broth and overcomes the bactericidal property of brilliant green dye (Winter et al., 2010).

Rappaport-Vassiliadis (RV) broth properties to promote *Salmonella* recovery is different, it uses $MgCl_2$ at concentrations that provides hypertonic conditions that inhibit Proteus and *Escherichia coli*, and malachite green to inhibit other competing bacteria (Rappaport et al., 1956; Vassiliadis et al., 1978; Peterz et al., 1989). RV presents a low pH which confers additional inhibition against coliforms and is well tolerated by *Salmonella* (Vassiliadis, 1983). Overall, $MgCl_2$, low pH and malachite green provide favorable conditions for *Salmonella* growth since a high osmotic pressure is created, low pH that delay bacterial metabolism, and inhibition of gram-positive organisms (Peterz et al., 1989).

2.6.5 Pre-enrichment alternatives

Examples of non-selective pre-enrichment broths used to isolate *Salmonella* from food include lactose broth, universal pre-enrichment, BPW, and trypticase soy broths; different studies highlighted the importance of resuscitating stressed and injured *Salmonella* and the use of non-

selective pre-enrichment broths to recuperate *Salmonella* from samples (Rappaport et al., 1956; D'Aoust & Maishment, 1979; D'Aoust, 1981; Ray, 1989; Chen et al., 2013). In the case of universal pre-enrichment broth (UPB), it was developed for the simultaneous proliferation of *Salmonella* and *Listeria* even though the first is gram-negative and the second gram-positive, UPB can be used to inoculate separate *Salmonella* and *Listeria* selective enrichment broths (Bailey & Cox, 1992).

Berrang et al. (2015) tested the buffering capacity of pre-enrichment media with different concentrations of buffer components. They reported the use of 1.0% peptone water buffered with sodium chloride (NaCl), disodium phosphate (Na_2HPO_4), sodium phosphate ($NaHPO_4$), 1 M tris pH 8, and sodium carbonate (Na_2CO_3) at different combinations. The combination of phosphate-tris-carbonate presented the best buffering chemistry having a pH drop at 24 h close to 6.5 in comparison to phosphate without Tris which suffered a pH drop at 24 h close to 5.2. The name given to this combination was “tris phosphate carbonate” (TPC). According to Jay (1998), pH 6.5 is near the ideal pH range reported for *Salmonella* growth.

In another study, Richardson et. al., (2021), showed that TPC had the best buffering capacity maintaining a near-neutral pH of a variety of ingredients and feed types during incubation among five pre-enrichment media tested (lactose broth “LB”, buffered peptone water “BPW”, double-strength buffered peptone water “2xBPW”, universal pre-enrichment broth “UPB”, and tris phosphate carbonate “TPC”).

2.6.6 Pre-enrichment considerations

Efforts have been made to enhance recovery of injured or stressed *Salmonella* from feed and food samples. The use of a non-selective media (pre-enrichment) before enrichment and isolation has been well studied and its importance is highlighted. An important consideration that Richardson et al. (2019) presented is that the pH impact on *Salmonella* was dependent on the

serotype and the stress status of the microorganism (liquid sample or dry sample). A pH value below 5.85 was enough to kill 50% of the *Salmonella* cells in a stressed state during incubation. Other authors mentioned that low pH alters the biochemical pathways of *Salmonella*.

Therefore, it is very important to provide injured and stressed *Salmonella* cells the opportunity to recover and multiply in a non-selective media (pre-enrichment). Tris phosphate carbonate (TPC) is an interesting pre-enrichment alternative that has a great buffering capacity allowing it to maintain a near-neutral pH in trials with a variety of ingredients and feed types. The importance of the use of a pre-enrichment in traditional culture methods to isolate *Salmonella* is presented in this document, however, efforts can be made to increase the effectiveness of this step with more evaluations of media. One factor to evaluate would be the response of some *Salmonella* serotypes to the buffering capacity of the media, time of incubation, temperature, and applicability (replication) in poultry industry laboratories.

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Chapter 3.0 Survey of feed mills around the United States for select bacterial pathogens

3.1 Introduction

Bacterial contamination in feed ingredients may occur during growing, harvesting, processing, storage, and distribution of feed, causing nutritional degradation if microflora persist (Maciorowski et al., 2007). Most microorganisms developed strategies to survive until there is enough water to strengthen their activity (Maciorowski et al., 2007). Several factors have participated in the contamination of animal and animal products, such as feed, associated fauna, water, animal manure handling, slaughtering, processing practices, and human related animal handling (Sofos, 2008). Contaminated feed ingredients and manufactured animal feed can be sources of non-endemic *Salmonella* serovars, pathogenic *Escherichia coli* (Gosling et al., 2021), *Escherichia coli* O157:H7 (Cox et al., 1983; Davies & Wray, 1997; Davis et al., 2003), *Clostridium perfringens* (Prió et al., 2001), and other pathogens of human health interest (e.g. *Campylobacter* species, *Listeria* monocytogenes) (Heredia & Garcia, 2018). The bacteria mentioned are part of the causes of enteric disorders in poultry (Hafez, 2011), and their monitoring is relevant because of the potential human health impact (Sapkota et al., 2007). Therefore, this study has as an objective to perform microbial analysis on feed ingredients and manufactured animal feed focusing on *Salmonella spp*., *E. coli*, and *Clostridium perfringens* to find possible sources of contamination of these bacteria.

3.2 Materials and methods

3.2.1. Feed sample collection

Samples were collected from 6 feed mills for this study (**Table 1**), letters A, B, C, D, E, and F were assigned. Feed mills A and B are integrators intended for swine and poultry production respectively, feed mills C, D, and F are toll mills (provide custom milling, mixing, and blending

services to other companies) intended for swine production, and feed mill E has research and education purposes. The samples collected (a total of 269) were ground corn, DDGS (distillers dried grains with solubles), wheat middlings, peanut meal, soybean meal, poultry by-product meal, meat and bone meal and manufactured feed which are post mixing, post cooling, mash loadout and pellet loadout. A "sample collection document for microbial analysis" (S.O.P) was implemented to standardize the samples, ground corn was collected from the ground corn silo and the other feed ingredients from storage containers or directly from trucks/trains during unloading. Manufactured feed samples were collected post mixing, post cooling (if pellet), and during loadout from different batches and with intervals of time. Representative samples of approximately 150 g were placed into sterile Whirl-Pak™ standard bags (Nasco®, Fort Atkinson, WI) and stored in coolers or a refrigerator until they were ready to be shipped to Auburn University. Once the samples were in Auburn University, they were stored in a cool room at 4 °C.

3.2.2. Microbiological Analysis

For microbiological analysis, Whirl-Pak™ blender filter bags (Nasco®, Fort Atkinson, WI), were used placing 10 g of each feed sample with 90 mL of Phosphate Buffered Saline (PBS; VWR, VWR Chemicals, Fountain Parkway, Solon, Ohio, USA) and stomached for 60 seconds for complete mixing, this first dilution was 1:10. Next, 1 mL of each bag (first dilution 10^{-1}) was transferred to an empty dilution tube and dilutions were performed by transferring 100 µL of the previous dilution into tubes with 900 µL of PBS until reach 10^{-5} dilution (vortex the tubes between dilutions).

For *Clostridium spp.* and *Salmonella spp.* isolation, 100 µL were spread plate by triplicate onto Tryptose Sulfite Cycloserine agar (TSC; Merck KGaA, EMID Millipore Corporation, Germany) and Xylose Lysine Tergitol 4 Agar (XLT4; Criterion™, Hardy Diagnostics, Santa

Maria, CA, USA) respectively. For *E. coli*, aerobic counts, and anaerobic counts, 10 µL were spot dropped by triplicate onto MacConkey agar (BD, Becton, Dickinson and Company, Sparks, MD, USA), Plate Count Agar (PCA; BD, Becton, Dickinson and Company, Sparks, MD, USA), and Anaerobic Agar (ANA; BD, Becton, Dickinson and Company, Sparks, MD, USA) respectively.

Salmonella spp. (XLT4), *E. coli* (MacConkey), and aerobic counts (PCA) plates were aerobically incubated for 24 hours at 37 °C. *Clostridium spp.* (TSC) and anaerobic counts (ANA) plates were anaerobically incubated for 24 – 48 hours in an anaerobic chamber (Bactron900, Anaerobic chamber, Shel Lab, Cornelius, OR, USA) at 37 C containing 5% CO_2, 5% H_2, and 90% N_2.

After incubation, all the plates were moved to a bench and individual differentiated colonies were enumerated and recorded according to the selective media agar (e.g. pink colonies are formed as an indicator of *E. coli* on MacConkey agar, and black colonies as an indicator of *Clostridium spp.* on TSC agar). From the black colonies identified using TSC agar, at least 4 single black colonies were transferred and streaked onto Trypticase™ Soy Agar with 5% sheep blood plates (TSA II + 5% SB; BD BBL™, Becton, Dickinson, and Company, Sparks, MD, USA) using a 1 µL disposable loop (VWR International, LLC, Radnor, PA) with the objective of visualization of hemolytic reactions. The plates were anaerobically incubated for 24 – 36 hours at 37 °C. From the isolates that presented double zone beta-hemolysis, which is a characteristic of *C. perfringens* alpha toxin (Dar et al., 2017), the colonies displaying this characteristic were cultured in Brain Heart Infusion Broth (BHIB; Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) with 20% glycerol to be frozen in sterile cryovials with beads at -80°C for further evaluation. Additionally, the isolates that presented either double zone beta-hemolysis or alpha-hemolysis, were transferred by streak plating onto TSC containing 5% Rabbit Blood (RB; Hemostat Laboratories, Dixon, CA,

USA) since the authors Gimenez & Ceccarelli (1970) and Suen et al. (1988), described that *C. argentinense* can produce beta-hemolytic colonies on rabbit blood agar while this ability is weak or absent on sheep blood agar. The isolates displaying the mentioned characteristics were also cultured in BHIB with 20% glycerol to be frozen in sterile cryovials with beads at -80°C for further evaluation.

Simultaneously to the transferring for dilutions, 1 mL of each bag of sample feed and PBS was transferred to 2 sets of 15 mL polypropylene centrifuge tubes containing 5 mL of BHIB, one set was incubated aerobically and the other anaerobically under the same conditions as the selective media agar plates for 24 – 48 hours. The purpose of culturing the samples in BHIB was to proliferate microorganisms that may not grow during regular plating using feed samples. For this reason, to confirm or discard the presence of bacteria, after incubation of BHIB, enriched cultures incubated aerobically were streaked onto MacConkey and PCA agar plates, and enriched cultures incubated anaerobically in TSC and ANA using 1 µL disposable loops, thereafter the plates were incubated again according to same conditions described before for the respective media agar. Following the criteria used by Munoz et al. (2021), samples that showed no growth (countable colonies) during regular plating, but after the streak plate of enrichment were positive, a value of 10 (1 $\log_{10}$) colony-forming unit was assigned. For *Salmonella spp.* evaluation, pre-enrichment and enrichment were used as described in **Chapter 4.0** of this document.

Microbial isolation was performed using selective media and colony counts are presented as follows: *Clostridium* spp. counts (**CSC**), *E. coli* counts (**ECC**), *Enterobacteriaceae* counts (**ENC**), aerobic counts (**AEC**), and anaerobic counts (**ANC**). In the case of MacConkey agar, which is a selective and differential media, general *Enterobacteriaceae* spp. grows on it, but *E. coli* presents pink colonies on this medium, for that reason ECC and ENC are reported as results.

3.2.3. Data Analysis

Colony forming units, were $\log_{10}$ transformed and analyzed using a generalized linear mixed model, Proc Glimmix (significant $P \leq 0.05$), means were separated by sample type using Tukey's HSD in SAS® 9.4 software. Additionally, correlations between the bacterial counts were performed using the CORR (Pearson correlation coefficient) procedure in SAS® 9.4 software. For the statistical analysis, samples were first divided into two groups, feed ingredients and manufactured feed. Then, ground corn which is the feed sample common in all the feed mills, was analyzed by feed mill. Feed ingredients were analyzed by sample type, and manufactured feed was analyzed by feed mill and for sample type.

3.3 Results and discussion

The summary of the mean $\log_{10}$ values of ground corn by feed mill for CSC, ECC, ENC, AEC, and ANC is in **Table 2**. Ground corn of feed mills A and B showed significant differences (P<0.05) with lower content of CSC, 0.30 and 0.63 respectively, compared to the other feed mills. Ground corn from feed mill C had lower ECC (1.27) and ENC (2.28), and ground corn from feed mills E and F presented lower ANC, 3.80 and 2.93 respectively.

The differences in bacterial content observed between ground corn of the different feed mills were dispersed with no clear tendencies, however it was interesting that ground corn from feed mill A had a numerically higher content of ECC (3.59) and ENC (4.71), but lower CSC (0.30) compared to the other feed mills, while ground corn from feed mill C had numerically lower content of ECC (1.27) and ENC (2.28), but higher CSC (2.42).

Table 3 summarizes the mean $\log_{10}$ values of the feed ingredients sampled by sample type. Differences (P<0.05) were observed among the feed ingredients where DDGS (distillers dried grains with solubles) and poultry by-product meal showed lower content of CSC (0.05 and

1.03 respectively), ECC (0.02 and 0.07 respectively), ENC (0.02 and 0.07 respectively), AEC (2.27 and 1.86 respectively), and ANC (1.58 and 1.57 respectively) compared to the other feed ingredients. Soybean meal presented no bacterial growth either in regular plating or after enrichment for CSC and ENC, and with DDGS and poultry by-product meal, displayed lower counts of ECC (0.14). Meat and bone meal was the feed ingredient with a higher level of CSC (4.56), and in general, more contaminated. According to Udhayavel et al. (2017), feed ingredients rich in animal proteins are the major source of *C. perfringens* contamination. Fishmeal or meat and bone meal in poultry feed increases the risk of necrotic enteritis (Kocher, 2003; Wu et al., 2014). These feed ingredients undergo high temperatures to destroy contaminant pathogens during the rendering process. However, *C. perfringens* is commonly found in its spore form, which is resistant to adverse environments such as thermal treatments (Casagrande et al., 2013). Meat and bone meal samples collected for this study had high levels of CSC compared to the other feed ingredients, even compared to poultry by-product meal since it is based on animal proteins, it could be expected to have high levels of CSC, but this was not the case. Therefore, contamination or re-contamination may be a possible reason for the high levels of CSC in meat and bone meal.

Overall, it is interesting to note that the three feed ingredients (DDGS, poultry by-product meal, and soybean meal) that show lower levels of contamination are by-products that involve a heat process. Distillers dried grains with solubles are a by-product of the bioethanol fermentation obtained from the dry milling industry and consist of undigested grains such as corn, wheat, sorghum, and barley (Iram et al., 2020; Buenavista et al., 2021). The process to obtain DDGS involves enzymes and high temperatures to break down starch components into glucose, fermentation by adding yeast cells, conversion of sugar into alcohol, and separation of ethanol and non-volatile components (whole stillage). Later the whole stillage is centrifugated resulting in a

liquid and solid fraction, thereafter the liquid fraction is condensed and combined with the solid fraction to produce DDGS (Kim et al., 2008; Buenavista et al., 2021). Temperatures used in dryers of ethanol plants can range from 93 to 232°C or more (Shurson et al., 2003) making this by-product primarily safer with low bacterial content.

Soybean meal is a by-product obtained after oil extraction from whole soybeans by either solvent extraction or using an extrusion-expeller (Pacheco et al., 2013). Soybean meal samples used in this study were the product of solvent extraction, this kind of extraction process typically involves the use of commercial hexane which generates a liquid and solid stream. The liquid stream is named "micella" and is composed of soybean oil and hexane, and the solid stream contains extracted soybean meal, hexane, residual oil, and water. Later the micella undergoes distillation which separates hexane from the oil, and the solid stream undergoes a desolventizing-toasting process (heat process) that separates hexane from the soybean meal cake, the temperature used in this process varies from 70 to 120°C and depends on the equipment used (Waldroup et al., 1985; Karr-Lilienthal et al., 2004; Paraíso et al., 2008).

According to the Association of American Feed Control Officials (AAFCO), poultry by-product meal is "the dry rendered product from a combination of clean flesh and skin with or without accompanying bone, derived from the parts of whole carcasses of poultry or a combination thereof, exclusive of feathers, heads, feet and entrails" and meat and bone meal is "the rendered product from mammal tissues, including bone, exclusive of any added blood, hair, hoof, horn, hide trimmings, manure, stomach and rumen contents except in such amounts as may occur unavoidably in good processing practices. It shall not contain extraneous materials not provided for by this definition" (AAFCO, 2022), the products name describes the composition, and this must correspond thereto. Poultry by-product meal is a popular protein source for poultry feed and

the pet food industry (Dozier III et al., 2003). Meat and bone meal is a good source of protein, amino acids, phosphorus, calcium, and energy, and can be used to feed species of livestock, poultry, and aquaculture, except ruminants (Ockerman & Basu, 2014). Rendering is a process that involves high temperatures systems to kill microorganisms and removes excess moisture from animal carcasses previously cut and ground, to be blended and cooked. The final step of this process is the separation of fat, protein materials, and wastewater, where the protein material is concentrated, dried, and ground. The result is what is known as poultry by-product meal or meat and bone meal, according to the type of rendering plant and kind of animal material used (Awonorin et al., 1995; Watson, 2006; Vidyarthi et al., 2021). As an example, the poultry by-product meal samples used in this study presented low bacterial content, while the meat and bone meal were the opposite. Feed ingredients may acquire bacteria at any time during transporting, processing, storage, and distribution (Maciorowski et al., 2007). Perhaps the transport or storage conditions for this specific batch of meat and bone meal were not adequate.

Burns et al. (2015) indicated that several raw materials used for pig feed manufacture contain high levels of *Enterobacteriaceae* counts and they are considered contaminated when the level was over 4 $\log_{10}$. Concerning the results presented, only wheat middlings (5.18) had *Enterobacteriaceae* counts over 4. The feed ingredients with higher content of ECC were wheat middlings (2.77), ground corn (2.58), and meat and bone meal (2.37). It has also been reported that animal feed ingredients like animal by-product meals and plant-based proteins can contain approximately 6 to 8 $\log_{10}$ microorganisms (Rechcigl, 1982; Davies & Hinton, 2000), however all the feed samples tested in this study were below 6. Another interesting consideration is that AEC is considered an unreliable index of microbial contamination since has no direct correlation with the occurrence of pathogens or toxins, however, unusually high levels of AEC can be considered

as potential contamination when above 6 to 7 $\log_{10}$ (Mendoca et al., 2020). None of the feed ingredients or manufactured feed presented AEC levels above 6.

Table 4 indicates the bacterial content for manufactured feed by the feed mill. Comparisons were made with samples collected post mixing (mash), post colling (after pelleting), and during loadout (pellet) in the case of feed mills A and E, and for feed mills C and D, which are intended for swine production, a comparison was made with samples collected post mixing (mash) and loadout (mash). No significative differences were observed in manufactured feed samples of feed mills C and D, and it can be inferred that the mixing process had no effect in decreasing bacterial load for these particular samples.

However, differences (P<0.05) were noted in the manufactured feed of feed mills A and E where the pelleting process was implemented with temperatures of 82.2 °C and 85.0 °C respectively. Post cooling and pellet loadout samples of feed mill A presented no bacterial growth with either regular plating or after enrichment for ECC and AEC, and the same samples had lower content of ANC (1.64 and 2.34 respectively) compared to post mixing (5.23). In the case of feed mill E, similar results were observed, post cooling and pellet loadout samples showed lower content of ECC (0.92 and 0.51 respectively) and ENC (0.89 and 0.31 respectively) compared to post mixing (3.31), nevertheless, pellet loadout samples had higher levels of CSC (3.46) compared to post cooling (2.41) and post mixing (1.25). The efficacy of the conditioning and pelleting process to reduce bacteria is dependent on temperature, retention time, and the microorganism of interest (Jones & Richardson, 2004). *C. perfringens* in manufactured feed is usually detected in its spore form, which is resistant to elevated (pelleting) or low temperatures, and to chemical preservatives (Sarker et al., 2000). According to Blank et al. (1996), post mixing samples of chicken feed had a range of 5.8 to 6.9 aerobic counts before conditioning and after conditioning a

range of 2.9 to 4.9. Samples from feed mills A and E had a range of 4.84 to 5.51 AEC before conditioning (pelleting) and after conditioning presented a range of 4.95 to 5.71. Based on these results and comparing with Blank et al. (1996), the conditioning (pelleting) was not effectively decreasing CSC and AEC in the manufactured samples tested.

The bacterial content for manufactured feed by sample type is presented in **Table 5.** It can be observed that post mixing samples had significant (P<0.05) higher levels of ECC (1.99) and ENC (3.62) compared to mash loadout, post cooling and pellet loadout samples. However, pellet loadout samples presented a higher content of CSC (2.76) compared to post mixing (1.41) and mash loadout (1.37). Like the results observed in **Table 4**, the pelleting process was effective in decreasing ECC between 1.65 to 2.4, and ENC between 3.0 to 4.6. Nevertheless, levels of CSC prevailed or even increased after a pelleting process which highlights the ability of this bacterium to produce spores that allows them to resist adverse environments and high temperatures (Heredia & Labbé, 2001), but also can be presumptive re-contamination concerning these samples and feed mill.

Correlation analysis was performed using the bacterial load of all the samples and the results are displayed in **Table 6**. It was expected to find positive correlations between ECC and ENC and between CSC and ANC since the first two grew in the same media agar and the other two were anaerobically incubated. For these results, weak positive correlation was considered with a r (Pearson coefficient) value between 0.25 to 0.50 and a moderate positive correlation between 0.50 to 0.75. All the correlations were significant (P<0.05). ECC and ENC presented a moderate positive correlation with a r value of 0.66, while ECC and ANC had a weak positive correlation (r 0.49). Additionally, ENC had a moderate positive correlation (r 0.63) with ANC and a weak positive correlation (r 0.43) with ANC. Surprisingly the correlation between CSC and AEC was

higher (*r* 0.47) compared to ANC (*r* 0.33), even though both are considered weak positive correlations. Lastly, AEC and ANC had a moderate positive correlation (*r* 0.50). It is interesting how in these results a tendency between ANC, ENC, and ECC was observed (**Table 3**, **4**, and **5**), most of the samples containing high ANC had also higher counts of ENC and ECC. Degelmann et al. (2009) indicated that *Enterobacteriaceae*, which is a facultative anaerobe, can outcompete obligate anaerobes when the environment lacks available oxygen. Anaerobically, *Enterobacteriaceae* degrade sugars to acetate, CO_2, H_2, formate, lactate, succinate, and ethanol (White, 2007). Since *Enterobacteriaceae* presents rapid growth in both aerobic and anaerobic conditions, ANC may be used as an indicator of *Enterobacteriaceae* contamination and indirectly for *E coli*. Van Schothorst & Oosterrom (1984), suggested that the level of *Enterobacteriaceae* counts is a good indicator of good manufacturing practices in rendering plants. Jones & Richardson (2004) and Maciorowski et al. (2004) suggested that high *Enterobacteriaceae* and *E. coli* counts in feed samples can be used as an indicator of contamination with *Salmonella* spp. Further research is necessary to explore and confirm the mentioned bacterial tendencies.

Lastly, nineteen samples that displayed either double zone beta-hemolysis or alpha-hemolysis on blood agar plates were isolated and stored using sterile cryovials with beads at -80°C for further evaluation.

3.4 Conclusions

1. Meat and bone meal was the feed ingredients with a higher degree of contamination especially *Clostridium* spp. levels compared to the other feed ingredients, while DDGS, poultry by-product meal and soybean meal were the feed ingredients displaying less bacterial content.

2. Based on these results, the pelleting process is effective at decreasing *E. coli* (1.65 – 2.10) and *Enterobacteriaceae* (3.00 – 4.60) levels, however *Clostridium* spp. can survive this process since they can form spores.
3. The correlation results suggest that high levels of anaerobic counts (ANC) are related to *Enterobacteriaceae* counts (ENC) and *E. coli* counts (ECC).
4. Nineteen isolates of presumptive *C. perfringens* or *C. argentinense* were isolated and stored for further analysis.
5. Animal feed can be a potential vector of pathogenic bacteria, and contaminated ingredients or manufactured feed can contaminate facility equipment resulting in cross-contamination. Contamination of feed ingredients may occur at any time during growing, harvesting, processing, transporting, handling, or storage, thus methods of control of bacteria in animal feed should be explored and evaluated.

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Table 1**.** Type of feed samples collected in different feed mills.

Feed mill	Type of feed mill	State	Type of feed sample	N of samples
A	Pigs, Integrator	OK	Ground corn	10
			Wheat middlings	10
			Post mixing	10
			Post cooling	10
			Pellet loadout	10
B	Broilers, Integrator	MS	Ground corn	8
C	Pigs, Toll Mill	IA	Ground corn	10
			DDGS	10
			Post mixing	10
			Mash loadout	8
D	Pigs, Toll Mill	IA	Ground corn	10
			DDGS	10
			Post mixing	10
			Mash loadout	10
E	Research and Education (R&E)	AL	Ground corn	14
			DDGS	14
			Poultry by-product meal	14
			Meat and bone meal	7
			Peanut meal	7
			Post mixing	14
			Post cooling	14
			Pellet loadout	14
F	Pigs, Toll Mill	IL	Ground corn	7
			DDGS	7
			Soybean meal	7
			Mash loadout	14

Abbreviations: Distillers dried grains with solubles (DDGS).

Table 2. Mean log_{10}/gram values of Ground corn by feed mill for *Clostridium* spp. counts (CSC), *E. coli* counts (ECC), *Enterobacteriaceae* counts (ENC), aerobic counts (AEC), and anaerobic counts (ANC).

Feed Mill	State	N	CSC	S.E.	ECC	S.E.	ENC	S.E.	AEC	S.E.	ANC	S.E.
A	OK	10	0.30^{c}	0.27	3.59^{a}	0.39	4.71^{a}	0.40	5.31^{a}	0.22	5.22^{a}	0.26
B	MS	8	0.63^{bc}	0.3	2.37^{ab}	0.44	3.31^{ab}	0.45	4.51^{ab}	0.25	4.97^{a}	0.29
C	IA	10	2.42^{a}	0.27	1.27^{b}	0.39	2.28^{b}	0.40	4.75^{ab}	0.22	4.29^{ab}	0.26
D	IA	10	1.65^{ab}	0.27	2.95^{a}	0.39	4.13^{a}	0.40	4.64^{ab}	0.22	4.70^{ab}	0.26
E	AL	14	2.39^{a}	0.23	2.93^{a}	0.33	3.74^{ab}	0.34	5.39^{a}	0.19	3.80^{bc}	0.22
F	IL	7	1.96^{a}	0.32	2.00^{a}	0.47	3.22^{ab}	0.48	4.02^{b}	0.27	2.93^{c}	0.31
P-value			< 0.0001		0.0022		0.0022		0.0008		< 0.0001	

[a-c] Values in columns with different superscripts are significantly different from each other ($P < 0.05$).
S.E.: Standard error.

Table 3. Mean log_{10}/gram values of feed ingredients by sample type for *Clostridium* spp. counts (CSC), *E. coli* counts (ECC), *Enterobacteriaceae* counts (ENC), aerobic counts (AEC), and anaerobic counts (ANC).

Type of Sample	N	CSC	S.E.	ECC	S.E.	ENC	S.E.	AEC	S.E.	ANC	S.E.
Ground corn	59	1.62^{bc}	0.11	2.58^{a}	0.14	3.60^{b}	0.13	4.86^{a}	0.16	4.33^{ab}	0.17
DDGS	41	0.05^{d}	0.13	0.02^{c}	0.17	0.02^{c}	0.16	2.27^{b}	0.19	1.58^{c}	0.20
Poultry by-product meal	14	1.03^{c}	0.23	0.07^{c}	0.28	0.07^{c}	0.27	1.86^{b}	0.32	1.57^{c}	0.34
Wheat middlings	10	2.28^{b}	0.27	2.77^{a}	0.34	5.18^{a}	0.32	5.62^{a}	0.38	5.57^{a}	0.40
Meat and bone meal	7	4.56^{a}	0.33	2.37^{ab}	0.40	3.34^{b}	0.38	5.27^{a}	0.45	4.58^{ab}	0.48
Peanut meal	7	1.60^{bc}	0.33	1.04^{bc}	0.40	2.92^{b}	0.38	4.98^{a}	0.45	3.83^{ab}	0.48
Soybean meal	7	0.00*	0.00	0.14^{c}	0.40	0.00*	0.00	4.62^{a}	0.45	3.09^{bc}	0.48
P-value		< 0.0001		< 0.0001		< 0.0001		< 0.0001		< 0.0001	

[a-d] Values in columns with different superscripts are significantly different from each other ($P < 0.05$).
S.E.: Standard error.
Abbreviations: DDGS (distillers dried grains with solubles).
*No colony-forming units were reported.

Table 4. Mean log_{10}/gram values of manufactured feed by feed mill for *Clostridium* spp. counts (CSC), *E. coli* counts (ECC), *Enterobacteriaceae* counts (ENC), aerobic counts (AEC), and anaerobic counts (ANC).

Feed Mill	P. T. °C	Bacteria	Feed Sample				S.E.			*P*-value
			Post mixing	Mash loadout	Post cooling	Pellet loadout				
A	82.2	#	10		10	10				
		CSC	0.93		1.54	1.79				
		ECC	2.47		0.00*	0.00*				
		ENC	4.66		0.00*	0.00*				
		AEC	5.51		4.95	5.49				
		ANC	5.23[a]		1.64[b]	2.34[b]	0.50	0.50	0.50	< 0.0001
C	-	#	10	8						
		CSC	2.04	1.71						
		ECC	2.05	1.79						
		ENC	3.30	3.87						
		AEC	4.94	5.12						
		ANC	4.37	4.71						
D	-	#	10	10						
		CSC	1.50	1.92						
		ECC	1.20	1.04						
		ENC	3.30	3.87						
		AEC	4.94	5.12						
		ANC	4.02	4.47						
E	85.0	#	14		14	14				
		CSC	1.25[c]		2.41[b]	3.46[a]	0.27	0.27	0.27	< 0.0001
		ECC	2.16[a]		0.92[b]	0.51[b]	0.35	0.35	0.35	0.0051
		ENC	3.31[a]		0.89[b]	0.31[b]	0.33	0.33	0.33	< 0.0001
		AEC	4.84		5.20	5.71				
		ANC	2.68		3.19	3.85				

[a-c] Values in columns with different superscripts are significantly different from each other ($P < 0.05$).
S.E.: Standard error.
P. T.: Pelleting temperature
#: Number of samples
*No colony-forming units were reported.

Table 5. Mean log_{10}/gram values of manufactured feed by sample type for *Clostridium* spp. counts (CSC), *E. coli* counts (ECC), *Enterobacteriaceae* counts (ENC), aerobic counts (AEC), and anaerobic counts (ANC).

Type of Sample	N	CSC	S.E.	ECC	S.E.	ENC	S.E.	AEC	S.E.	ANC	S.E.
Post mixing	44	1.41[b]	0.17	1.99[a]	0.19	3.62[a]	0.24	4.96[a]	0.15	3.95[a]	0.27
Mash loadout	32	1.37[b]	0.21	1.12[b]	0.22	2.32[b]	0.28	4.18[b]	0.18	3.41[ab]	0.32
Post cooling	24	2.05[ab]	0.24	0.54[b]	0.25	0.52[c]	0.32	5.09[a]	0.21	2.54[b]	0.37
Pellet loadout	24	2.76[a]	0.24	0.30[b]	0.25	0.18[c]	0.32	5.62[a]	0.21	3.22[ab]	0.37
P-value		< 0.0001		< 0.0001		< 0.0001		< 0.0001		0.0235	

[a-c] Values in columns with different superscripts are significantly different from each other ($P < 0.05$).
S.E.: Standard error.

Table 6. Correlations between five different bacterial counts.

Colony-forming counts		*Clostridium* spp.	*E. coli*	*Enterobacteriaceae*	Aerobic	Anaerobic
Clostridium spp.	*r*	1.00	0.19	0.22	0.47	0.33
	P-value		0.0019	0.0002	<.0001	<.0001
E. coli	*r*	0.19	1.00	0.66	0.36	0.49
	P-value	0.0019		<.0001	<.0001	<.0001
Enterobacteriaceae	*r*	0.22	0.66	1.00	0.43	0.63
	P-value	0.0002	<.0001		<.0001	<.0001
Aerobic	*r*	0.47	0.36	0.43	1.00	0.50
	P-value	<.0001	<.0001	<.0001		<.0001
Anaerobic	*r*	0.33	0.49	0.63	0.50	1.00
	P-value	<.0001	<.0001	<.0001	<.0001	

r = Pearson correlation coefficient, Number of samples: 269.
P -value: < 0.05 means correlation was significant.

Chapter 4.0 Evaluation of Tris Phosphate Carbonate (TPC) as a pre-enrichment method to recover *Salmonella*, compared to Buffered Peptone Water (BPW)

4.1 Introduction

Detecting particular microorganisms from specific sources can be difficult and proper consideration must be given to the influence of environmental factors before detection (Bissonnette et al., 1975). Exposure to freezing, heating, or freeze-dying negatively impacts the attempt at detection and enumeration of some microorganisms because of physiologically weakened or injured cells (Clark & Ordal, 1969; Ray et al., 1971; Ray & Speck, 1973). The type of sample, level of stress or injury applied by the sample matrix or environment, presence of commensal bacteria, and level of *Salmonella* in the sample matrix are important considerations for the successful recovery of this bacteria (Busse, 1995). Most bacteria, if not all, face life-threatening and hostile conditions in their natural habitats, such as oxidations, heavy metals, DNA-damaging agents, osmolarity, starvations, weak acids, and a wide range of temperatures or pH values other than the optimal for growth (Foster, 1995). The optimal temperature for *Salmonella* growth is 37°C with a range of 5- 47°C, and the optimum pH is 6.5-7 with a range of 4 to 9 (Jay, 1998; Li et al., 2012; Bhunia, 2018).

Poultry feed is a potential source of *Salmonella* (Cox et al., 1983; Quinn et al., 1995; Veldman et al., 1995; Davies & Wray, 1996; Heyndrickx, et al., 2002; Munoz et al., 2021). However, a low percentage of samples tested are reported as positive for *Salmonella* (Heyndrickx et al., 2002; Veldman et al., 1995). Standard cultural procedures for the isolation of *Salmonella* generally include pre-enrichment of samples in a non-selective broth medium, enrichment in a selective broth medium, isolation by presumptive screening and serological confirmation of presumptive isolates (D'Aoust, 1981). The use of selective media may hinder the detection of such

stressed or injured microorganisms since injured cells become sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies (Bissonnette et al., 1975).

A pre-enrichment medium should not only be a noninhibitor for *Salmonella* but should also be capable of supporting the proliferation of small numbers of these bacteria especially in higher dilutions where any possible nutritional or inhibitory substances from the feed being tested should have been diluted out (North, 1961). For example, according to the HIMEDIA® technical data sheet (HiMedia Laboratories, 2022), BPW is a pre-enrichment medium intended to support the recovery of sub-lethally harmed *Salmonella* before transfer to a selective medium. This medium presents no inhibitors, is well buffered, and offers conditions for revival of cells injured by food preservation processes.

Berrang et al. (2015) tested the buffering capacity of pre-enrichment media with different concentrations of buffer components. They reported the use of 1.0% peptone water buffered with sodium chloride (NaCl), disodium phosphate (Na_2HPO_4), sodium phosphate ($NaHPO_4$), 1 M tris pH 8, and sodium carbonate (Na_2CO_3) at different combinations. The combination "tris phosphate carbonate", which was named TPC, presented the best buffering chemistry having a pH drop at 24 h close to 6.5 in comparison to phosphate without Tris which suffered a pH drop at 24 h close to 5.2.

In another study, Richardson et al. (2021) showed that TPC had the best buffering capacity maintaining a near-neutral pH on a variety of ingredients and feed types during incubation among five pre-enrichment media tested (lactose broth "LB", buffered peptone water "BPW", double-strength buffered peptone water "2xBPW", universal pre-enrichment broth "UPB", and tris phosphate carbonate "TPC"). In the previous studies mentioned, manufactured feed and feed ingredients, mainly for poultry production, were tested using different pre-enrichments and

observing how pH changed after incubation. The buffer capacity of TPC was highlighted among other pre-enrichments. However, the samples were not tested for *Salmonella* by applying further steps like enrichment, plating in selective agar, agglutination test, or biochemical confirmation. BPW is the standard pre-enrichment used for recovery of *Salmonella* in feed samples, therefore, based on the previous information, the aims of this project were to evaluate the buffer capacity of TPC and BPW using manufactured feed and feed ingredients, and evaluate TPC as a pre-enrichment method to recover *Salmonella* compared to BPW. Additionally, microbiological isolation and identification of possible *Salmonella* serovars.

4.2 Materials and methods

Feed samples from **Chapter 3.0** (Survey of feed mills around the United States for select bacterial pathogens) were processed simultaneously for this study (**Table 1)**. The TPC formula (**Table 7**) was consulted with the authors and adapted from Berrang et al. (2015). All solid components were added into a sterilized beaker with 600 mL of deionized water and mixed, then 100 mL of Tris pH 8.0 was added and finally more deionized water to bring the final volume to 1 L. The pH of the TPC solution was measured and adjusted using drops of 6N Hydrochloric Acid (HCl) to reach a final pH of 8.0. Lastly, the TPC solution was sterilized using a Corning sterile filter system of 0.22 μm (Corning Incorporated, Corning, NY). The initial pH of BPW (BD Difco, Franklin Lakes, NJ) solution was 7.0.

From each feed sample, 2.5 g of sample was added to 22.5 mL of TPC and BPW in 50 mL polypropylene centrifuge tubes, vortexed with a VWR® vortex mixer for 30 sec and then incubated for 24 h at 37°C. The initial pH (0 h) was measured with a VWR sympHony B10P benchtop pH meter (VWR Chemicals, Fountain Pkwy, OH) after mixing and the final pH (24 h) was measured after incubation. After incubation, 1 mL of the samples was transferred to 5 mL of

tetrathionate brilliant green broth (HiMedia Laboratories, West Chester, PA) in 15 mL polypropylene centrifuge tubes and incubated for 24 h at 37°C. Thereafter, all the samples were streak plated onto Xylose Lysine Tergitol 4 agar (XLT4: Criterion™, Hardy Diagnostics, Santa Maria, CA, USA) and incubated for 24 h at 37°C for subsequent visual identification of typical black "fish-eye" *Salmonella* colonies associated with this media. From the suspect *Salmonella* samples in the media agar, single colonies found were transferred (streak plate) to another XLT4 plate and incubated. Next, after a second visual confirmation, a single colony was transferred to a *Salmonella* ChromoSelect agar (Sigma-Aldrich Corporation, St. Louis, MO) and incubated for 24 h at 37°C. With the visual confirmation in the ChromoSelect agar, a single colony from the plate was inoculated onto Triple Sugar Iron (TSI) (BD Difco, Franklin Lakes, NJ), Lysine Iron Agar (LIA) (BD Difco, Franklin Lakes, NJ), and Urea Agar slants (BD Difco, Franklin Lakes, NJ) and incubated for 24 h at 37°C for biochemical confirmation.

From the same samples on the ChromoSelect agar, an agglutination test for serological confirmation was performed using polyvalent serum A-V for *Salmonella* spp. (Difco, BD), then, based on serology from *Salmonella* antiserum Poly Groups A, B, C, D, and E (Difco, BD) and antiserum B, C_1, C_2, D_1, E, or K (Difco, BD), sero-grouped. Next, after agglutination testing, the isolates were shipped to the National Veterinary Services Laboratory in Ames, IA for serovar characterization.

4.2.1 Data Analysis

Data were analyzed using a generalized linear mixed model, Proc Glimmix (significant $P \leq 0.05$), means were separated by sample type using Tukey's HSD and pH change in time (initial–final) was analyzed using dependent (paired) *t*-test for each sample type in SAS® 9.4 software.

4.3 Results and discussion

The average initial and final pH of each feed type using both pre-enrichments is presented in **Table 8**. The initial pH of the samples with TPC was above neutral pH (~7.0) while the samples with BPW yielded mixed values. Conversely, the final pH (after 24 h incubation) of the samples with TPC was in a range of 5.75 to 7.09 while the samples with BPW reported values below pH 6.5. According to Jay (1998), pH 6.5 is near the ideal pH range for *Salmonella* growth. Berrang et al. (2015) reported a pH close to 6.5 after 24 h incubation using TPC in standard broiler feed highlighting the buffering chemistry capacity of this pre-enrichment. Richardson et al. (2019) reported that the pH impact on *Salmonella* was dependent on the serotype and the stress status of the microorganism (liquid sample or dry sample). They showed that pH 4.85 was required to kill 50% of *S.* Typhimurium in a non-stressed or liquid state during 24 h incubation, while in a stressed state, a pH of 5.85 was low enough to kill 50% of the cells.

Differences were observed comparing pH values by sample type ($P < 0.001$); for initial TPC pH, DDGS (6.98) and poultry by-product meal (7.60) presented lower pH values; moreover, for final TPC pH readings, meat and bone meal (7.77) presented the higher pH value while peanut meal (5.75) and soybean meal (5.59) the lower compared to the other feed types. Initial BPW pH readings showed that DDGS (5.81) and poultry by-product meal (6.54) also presented lower pH values. Furthermore, for final BPW pH, meat and bone meal (6.45) presented a higher pH value; however, pellet loadout (5.05), post cooling (5.11), wheat middlings (5.15), and soybean meal (4.86) presented lower pH values. Richardson et. al. (2021) reported similar pH values after 24 h incubation using TPC and BPW for meat and bone meal of 7.88 and 6.59 respectively. Cox et al. (2013) reported pH values of 4.3 for DDGS, 4.8 for ground corn and 4.6 for wheat middlings after 24 h incubation using BPW, which differs from the data presented here (5.90, 6.01, and 5.15

respectively). It is important to remark that DDGS and wheat middlings are by-products that vary in composition since the raw material used can be from different varieties, different environmental conditions, and the techniques to obtain the mentioned by-products can be different (Cromwell et al., 2000; Belyea et al., 2010). For example, DDGS are obtained by two main techniques: dry grind processing and wet milling (Rausch & Belyea, 2006). Wheat middlings are obtained by mechanical and pneumatic methods applied to separate endosperm particles from the germ and bran; as a result, several fractions in different amounts are obtained such as screenings, bran, middlings, shorts, and red dog (Poore et al., 2002). The pH values reported by Cox et al. (2013), may differ because different sources of feed ingredients were used for their experiment.

The difference between initial and final mean pH values expressed in percentage using pre-enrichment media TPC and BPW is presented in **Table 9**. Wheat middlings, peanut meal, soybean meal and manufactured feed, which includes pellet loadout, post cooling, post mixing, and mash loadout presented the higher pH difference after 24 h incubation with a drop of at least 17% to 32% with both pre-enrichments. Wheat middlings contain highly fermentable carbohydrates (ZoBell et al., 2003), peanut meal and soybean meal are excellent sources of protein (Batal et al., 2005; Dozier III & Hess, 2011), and finished feed is manufactured to ensure that the animal receives all the required nutrients and supplements (Huss et al., 2018). Therefore, high bacterial activity (fermentation) was expected during incubation using non-selective pre-enrichments. The common end-products of bacterial fermentation are lactic acid, formic acid, acetic acid, butyric acid, butyl alcohol, acetone, ethyl alcohol, carbon dioxide, and hydrogen, which will all decrease the pH of the medium (Müller, 2001).

Using a dependent (paired) *t*-test, highly significant differences ($P<0.001$) were found in most of the feed ingredients but not DDGS. This means that the set of pH values initial and final

was significantly different from each other for the mentioned feed samples. Is also important to note that DDGS samples maintained the pH after incubation with a slight drop using TPC (6.98 to 6.91) and a slight increase using BPW (5.81 to 5.90).

DDGS are a co-product of ethanol-producing factories that utilize corn and wheat as raw materials that contain high levels of crude protein, oil, and fiber (Abd El-Hack et al., 2019). DDGS fiber is composed mostly of cellulose, hemicelluloses, lignin, and pectin which are complex carbohydrates that are not precisely easy to break (Lamsal et al., 2012). It is also known that DDGS are used as a fermentation feedstock, but pre-treatment methods are needed to break down the lignin impediment which is recalcitrant to bacterial attack and can result in the release of inhibitory products for microbes (Vicuña, 1988; Iram et al., 2020). DDGS also contain sulfuric acid that is used for the control of pH during ethanol fermentation and the cleaning process of bioethanol production (Wu et al., 2015). Sulfuric acid can inhibit or even kill microorganisms including bacteria and fungi due to its strong oxidizing corrosiveness (Wang et al., 2018). Perhaps the combination of the characteristics of DDGS affected the bacterial activity during incubation (degradation) resulting in almost no pH change.

An important consideration regarding pH and *Salmonella* spp. grow is the Acid Tolerance Response (ATR). Normally, a cell maintains its internal pH (pH_i) through pH homeostasis over different external pH values (pH_o). However, it has been shown that *Salmonella* species can develop mechanisms that protect cells against strong acid environments (pH 3.0 to 4.0) when pH homeostasis fails (Foster & Hall 1991). ATR is the mechanism that enables *Salmonella* species to survive in extreme acid conditions like the gastrointestinal tract (Yuk &Schneider, 2006). Two pH-dependent ATR systems have been described in studies with *S.* Typhimurium, the first is ATR which occurs when some proteins are induced after exposure to pH 5.8 (pre-acid shock) and others

after exposure to pH 4.5 or below (acid shock). The function of these proteins is to enhance the survival of the bacterium by inducing pH homeostasis and repairing or preventing acid damage to macromolecules (Foster, 1991). The second system is stationary-phase ATR where proteins different than log-phase acid shock proteins are synthesized at pH 4.3 but in a sustained way for several hours, which appears to confer protection against acid stress independently of the pH of the medium (Lee et al., 1994). The mentioned proteins are called acid shock proteins (ASPs). Several genes and proteins are involved in the mechanism of bacterial ATR such as RpoS which is an RNA polymerase sigma subunit and is a product of stress response. In addition to this protein being transcribed the cell can show modifications in its membrane fluidity and fatty acid composition (Brown et al., 1997; Nazir et al., 2019). The results of Foster (1995) showed that organisms (*S.* Typhimurium) first adapted to a moderated acid pH can tolerate acidity below pH 4.0 for longer periods compared to those that were not previously adapted since general stress defense mechanisms were induced by acid shock. Concerning the samples tested for this study, they were not exposed to acid pH environments and the lower pH values after incubation observed were 5.59 (soybean meal, using TPC) and 4.68 (peanut meal, using BPW), **Table 8**. However, it is unknown how severe the treatments (stress) are applied to the different feed samples before arriving at the laboratory, for example drying of grains, milling, pelleting, rendering processes, or chemical treatments for by-products. There is evidence that *Salmonella* serovars respond differently to sub-lethal stresses, and those survival cells may present greater resistance to further treatments than untreated cells (Clemente-Carazo et al., 2021).

All the feed samples tested were negative for *Salmonella*, except for meat and bone meal. Several researchers state that the detection of *Salmonella* in feed during pre-enrichment or enrichment can be negatively affected by the level of background microflora (Koyuncu &

Haggblom, 2009; Kuijpers & Mooijman, 2012; Mooijman, 2018). From meat and bone meal samples, four were positive for *Salmonella* (**Table 10**), three samples using BPW and one using TPC. The isolates were identified as *Salmonella* Oranienburg, *S.* Senftenberg, *S.* Agona, and *S.* Infantis **(Table 10).**

S. Oranienburg serovar is a subspecies of *Salmonella enterica* and can cause illness in humans (Medrano-Félix et al., 2013). The Centers for Disease Control and Prevention (CDC) have several reports linked to this serovar. In 2015, an outbreak of eight people from three states infected with *S.* Oranienburg was related to an egg company ending in a recall; the last report made by the CDC was to follow food safety steps to handle and cook eggs safely (CDC, 2016a). In 2022, an outbreak involving 1,040 people infected with *S.* Oranienburg was linked to the consumption of onions from Mexico (CDC, 2022). *S.* Senftenberg is a nontyphoidal serovar (NTS) and, like most *Salmonella* serovars, can persist in feed mills and poultry farms even after cleaning and disinfection procedures (Broennum Pedersen et al., 2008). It is usually isolated from feed raw materials, environmental samples taken in the feed mill and poultry houses, and the processing plants (Bailey et al., 2001; Nesse et al., 2003; Broennum Pedersen et al., 2008). *S.* Senftenberg can cause illness in humans, and several reports of foodborne outbreaks were linked to this serovar (L'Ecuyer et al., 1996; Rushdy et al., 1998; Hu et al., 2008). *S.* Agona was one of the fifteen most common serotypes in human infections in 2016 in the European Union (European Food Safety Authority, EFSA, 2017). *S.* Agona strains isolated from chicken carcasses have been identified as strong biofilm producers (Díez-García et al., 2012). In 2008, the CDC reported an outbreak of *S.* Agona infection linked to rice and wheat puff cereal (CDC, 2008). *S.* Infantis can infect humans and numerous animal species. This serovar mainly affects children and can persist over a long period (Ranjbar et al., 2012). In 2019, the CDC reported a multistate outbreak of *S.* Infantis

infections related to raw chicken products. According to the National Enteric Disease Surveillance: *Salmonella* Annual Report, 2016 (CDC, 2016b), and the Food Safety and Inspection Services (FSIS, 2014), among twenty serotypes that includes *S.* Enteritidis (#1), *S.* Typhimurium (#3), *S.* Heidelberg (#12), *S.* Oranienburg (#13), and *S.* Agona (#18), *S.* Infantis was the sixth most frequently reported serotype isolated from poultry products in the United States.

The four serovars identified are common in poultry production and can cause illness in humans but do not cause sickness in birds. However, birds can act as reservoirs of these bacteria (Kallapura et al., 2014; Kamble & Lee, 2016; Myoujin et al., 2003).

Meat and bone meal samples belonged to the same batch, however the samples were taken from different locations. The final pH of the samples was in a range of 6.40 to 6.45 using BPW and 7.78 using TPC. Meat and bone meal is a by-product obtained from the rendering industry after a process of cooking mammal carcasses, eliminating fat, and drying and crushing (Cascarosa et al., 2012). The rendering process includes a heat step that kills any microorganisms, in consequence contamination with *Salmonella* is most unlikely from processing (Jiang, 2016). Rendered products and finished feed are most likely to be contaminated with *Salmonella* from rodents and fomites in the spaces of the processing plants and feed mills (Fedorka-Cray et al., 1997) or during storage or transportation to other locations. *Salmonella* can survive long periods in dried products, such as animal feed (Beuchat et al., 2013). A survey covering 1 year (2010) tested a variety of render and blender operations across the United States and Canada showed that the contamination rate for *Salmonella* in the rendered animal meals produced in North America was 8% (Jiang, 2016). Therefore, special attention must be given to avoid recontamination during transportation, storage, handling, and sampling regarding this feed ingredient (in this particular

case) and other feed ingredients. It is essential to apply good hygiene practices in the feed mill environment to reduce the prevalence of *Salmonella* (Parker et al., 2022).

4.4 Conclusions

1. TPC provided greater buffer capacity towards neutral pH compared to BPW.
2. Manufactured feed samples (post mixing, post cooling, pellet loadout, and mash loadout), wheat middlings, peanut meal, and soybean meal experienced a pH drop after 24 h incubation of at least 17% to 32%.
3. Three *Salmonella* isolates were recovered from meat and bone meal samples using pre-enrichment BPW, compared to one in TPC.
4. Further research of feed inoculated with a known *Salmonella* strain and concentration and essayed with pre-enrichment TPC and BPW is necessary to determine their efficacy in recovering *Salmonella.*

4.5 References

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Table 7. Tris phosphate carbonate (TPC) formula.

TPC Formula	Brand	Amount (1 L)
Peptone	BD Bacto, Franklin Lakes, NJ.	10 g (1%)
NaCl (Sodium Chloride)	VWR Chemicals, Fountain Pkwy, OH.	5 g (0.085 M)
Na_2HPO_4 (Disodium phosphate)	VWR Chemicals, Fountain Pkwy, OH.	3 g (25 mM)
$NaHPO_4$ (Sodium phosphate)	Fisher Scientific, Fair Lawn, NJ.	1.5 g (11 mM)
Na_2CO_3 (Sodium carbonate)	Fisher Scientific, Fair Lawn, NJ.	4.2 g (50mM)
1 M Tris, pH 8.0	VWR Chemicals, Fountain Pkwy, OH.	100 mL (100 mM)
H_2O	-	~ 900 mL

Adapted from Berrang et al., 2015.

Table 8. Mean separation of pH values by feed type using pre-enrichment media TPC and BPW.

Feed type	N of Samples	TPC (pH)				BPW (pH)			
		Initial	S.E.	Final	S.E.	Initial	S.E.	Final	S.E.
Ground corn	59	8.03[a]	0.02	7.14[b]	0.04	7.05[a]	0.02	6.01[ab]	0.05
DDGS	41	6.98[d]	0.02	6.91[cd]	0.05	5.81[d]	0.03	5.90[bc]	0.06
Poultry by-product meal	14	7.60[c]	0.04	7.14[bc]	0.08	6.54[c]	0.05	6.22[ab]	0.1
Wheat middlings	10	7.94[ab]	0.05	6.57[ef]	0.09	7.09[a]	0.06	5.15[d]	0.12
Meat and bone meal	7	7.85[ab]	0.06	7.77[a]	0.11	6.96[ab]	0.07	6.45[a]	0.15
Peanut meal	7	8.04[a]	0.06	5.75[g]	0.11	6.92[ab]	0.07	4.68[d]	0.15
Soybean meal	7	7.93[ab]	0.06	5.59[g]	0.11	6.99[ab]	0.07	4.86[d]	0.15
Post mixing	44	7.99[a]	0.02	6.54[f]	0.04	6.91[ab]	0.03	5.69[c]	0.06
Mash loadout	32	7.78[b]	0.03	6.80[de]	0.05	6.82[b]	0.03	5.93[abc]	0.07
Post cooling	24	8.04[a]	0.03	6.39[f]	0.06	6.92[ab]	0.04	5.11[d]	0.08
Pellet loadout	24	8.02[a]	0.03	6.23[f]	0.06	6.87[ab]	0.04	5.05[d]	0.08
P-value		< 0.0001		< 0.0001		< 0.0001		< 0.0001	

[a-f] Values in columns with different superscripts are significantly different from each other ($P < 0.05$).
S.E.: Standard error.
Abbreviations: DDGS (distillers dried grains with solubles).

Table 9**.** Dependent *t*-test for the pH difference before and after incubation (24 h) of feed ingredients and manufactured feed using pre-enrichment media TPC and BPW.

Type of Sample	N of Samples	TPC (pH)					BPW (pH)				
		Initial	Final	I - F (%)	S.E.	*P*-Value	Initial	Final	I - F (%)	S.E.	*P*-Value
Ground corn	59	8.03*	7.14*	11.11%	0.03	< 0.0001	7.05*	6.01*	14.64%	0.07	< 0.0001
DDGS	41	6.98	6.91	1.01%	0.04	0.076	5.81	5.90	-1.55%	0.06	0.164
Poultry by-product meal	14	7.60*	7.14*	6.08%	0.06	< 0.0001	6.54*	6.22*	4.89%	0.02	< 0.0001
Wheat middlings	10	7.94*	6.57*	17.25%	0.06	< 0.0001	7.09*	5.15*	27.40%	0.06	< 0.0001
Meat and bone meal	7	7.85*	7.77*	1.06%	0.02	0.004	6.96*	6.45*	7.33%	0.03	< 0.0001
Peanut meal	7	8.04*	5.75*	28.46%	0.13	< 0.0001	6.92*	4.68*	32.36%	0.16	< 0.0001
Soybean meal	7	7.93*	5.59*	29.48%	0.13	< 0.0001	6.99*	4.86*	30.46%	0.09	< 0.0001
Post mixing	44	7.99*	6.54*	18.20%	0.03	< 0.0001	6.91*	5.69*	17.68%	0.06	< 0.0001
Mash loadout	32	7.78*	6.80*	12.55%	0.11	< 0.0001	6.82*	5.93*	13.03%	0.09	< 0.0001
Post cooling	24	8.04*	6.39*	20.52%	0.05	< 0.0001	6.92*	5.11*	26.18%	0.03	< 0.0001
Pellet loadout	24	8.02*	6.23*	22.22%	0.06	< 0.0001	6.87*	5.05*	26.49%	0.05	< 0.0001

S.E.: Standard error of the difference scores.
*Means of the two sets of pH values with an asterisk are significantly different from each other (P <0.05).
I – F (%): Difference (initial minus final) of initial and final mean values (pH) expressed in percentage.
Abbreviations: DDGS (distillers dried grains with solubles).

Table 10. *Salmonella* isolates recovered using pre-enrichment TPC and BPW.

Sample	P.E.	pH		Selective agar	Agglutination test		B.C.	Serotype
		Initial	Final		Poly	Group		
Meat and bone meal	BPW	6.89	6.40	Positive	A	C1	Positive	Oranienburg
	BPW	6.89	6.45	Positive	B	E	Positive	Senftenberg
	BPW	7.09	6.42	Positive	A	B	Positive	Agona
Meat and bone meal	TPC	7.80	7.78	Positive	A	C1	Positive	Infantis

P.E.: Pre-enrichment
Selective agar: Xylose Lysine Tergitol 4 agar (XLT4) and HiCrome (ChromoSelect agar)
B.C.: Biochemical confirmation

Chapter 5.0 Summary, conclusions, and future implications

Animal feeds are intended to fulfill animal's nutritional requirements for maintenance, activity, production, and reproduction. Nevertheless, humans are the consumers of animal-based products such as meat, milk, or eggs, therefore, animal feeds are considered part of the human food chain and its safety should be contemplated for both animals that eat it and for the final consumer (Crawshaw, 2012). Feed ingredients can be source for non-endemic *Salmonella* serovars and other enteric bacteria, including pathogenic *Escherichia coli* (Gosling et al., 2021), and *Clostridium perfringens* (Prió et al., 2001). Enteric health and nutrition (feed) are closely related, and the mentioned bacteria are part of the causes of enteric disorders in poultry (Hafez, 2011).

Based on the findings of Chapter 3.0, feed ingredients vary in bacterial levels, however DDGS, poultry by-product meal, soybean meal were the feed ingredients with less bacterial content while meat and bone meal had the higher bacterial content, especially *Clostridium* spp. counts (CSC). Manufactured feed samples that were pelleted showed a decrease in *E. coli* counts (ECC) between 1.65 - 2.10 $\log_{10}$, and *Enterobacteriaceae* counts (ENC) between 3.00 - 4.60. Nevertheless, CSC remained the same or even more after this process. *Clostridium* spp. can survive heat treatments since they are able to form spores. Correlations made between the different bacterial counts suggests that high levels of anaerobic counts (ANC) are related with ENC and ECC.

Animal feed can be a potential vector of pathogenic bacteria, and contaminated ingredients or manufactured feed can contaminate facility equipment resulting in cross-contamination of other feed ingredients. Contamination of feed ingredients may occur any time during growing, harvesting, processing, transporting, handling, or storage, thus methods of control of bacteria in animal feed should be explored and evaluated.

Based on the findings of Chapter 4.0, pre-enrichment TPC provide greater buffer capacity towards neutral pH compared to BPW. However, three *Salmonella* isolates were recovered from meat and bone meal samples using BPW and one using TPC. The serovars identified were *S.* Oranienburg, *S.* Senftenberg, and *S.* Agona from BPW, and *S.* Infantis from TPC. Manufactured feed samples which are post mixing, post cooling, pellet loadout and mash loadout, and wheat meddling, peanut meal, and soybean meal were samples that experienced a pH drop of at least 17% to 32% after 24 h incubation.

Further research is needed to determine tendences of how pH change according to the type of sample (ingredient or manufactured feed). Additionally, evaluation of *Salmonella* recovery capacity of these two pre-enrichments using feed inoculated with a known concentration of a *Salmonella* strain previously identified like *S.* Infantis, which importance has increased the last years becoming one of the top 10 serotypes causing human illness in the United States (CDC, 2016).

5.1 References

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