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PREVALENCE and BIOLOGICAL CONTROL of SALMONELLA CONTAMINATION in RENDERING PLANT ENVIRONMENTS and the FINISHED RENDERED MEALS

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PREVALENCE and BIOLOGICAL CONTROL of *SALMONELLA* CONTAMINATION in RENDERING PLANT ENVIRONMENTS and the FINISHED RENDERED MEALS

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ABSTRACT

Although the rendering process serves as invaluable means for the disposal of inedible animal by-products, the finished products often harbor pathogenic and opportunistic microorganisms such as *Salmonella* and enterococci, respectively. The temperatures used during the rendering process far exceed the heat tolerance threshold of most bacterial species, so cross-contamination from the environment and/or from the incoming raw material is the proposed source of the contamination. Research has demonstrated that the raw material coming into the rendering facility is highly contaminated with pathogenic bacteria including *Salmonella*. While not in a rendering facility, studies have also demonstrated that bacteria such as *Salmonella* can persist on food processing equipment and be transferred into the product upon contact. The objectives of this study were to: 1) isolate and characterize *Salmonella* and enterococci from finished animal by-products, 2) produce and optimize a bacteriophage cocktail against *Salmonella*, 3) apply the bacteriophages to reduce *Salmonella* levels on environmental surfaces found in a rendering facility and in raw offal, and 4) use the bacteriophage as a feed additive to reduce or prevent *Salmonella* infection in mice.

To determine the prevalence of *Salmonella* and enterococci, two hundred finished meals provided by various rendering facilities across the U.S. were analyzed. While the animal meals were shown to not be a suitable environment for bacterial growth (moisture content 1.9 to 11.5%), these products did contain enterococci and *Salmonella*. Enterococci were detected in 83% of the samples and accounted for up to 54% of the total bacterial count, which ranged from 1.7 to 6.8 log CFU/g. Characterization of the

enterococci isolates revealed that only 3 isolates were resistant to vancomycin (32 µg/ml). PCR analysis revealed that none of these 3 VRE isolates were *E. faecalis* or *E. faecium*. In addition, no VRE isolates were of the *VanA* or *VanB*-type, which confer the highest levels of resistance to vancomycin. *Salmonella* (n = ?) was isolated from 8.7% of the finished meal samples. There were 13 *Salmonella* serotypes identified among the isolates with 16 pulse field gel electrophoresis (PFGE) patterns. Thermal tolerance studies revealed that these *Salmonella* isolates had D-values of 9.27-9.99, 2.07-2.28, and 0.35-0.40 min at 55°C, 60°C, and 65°C, respectively.

As a means to prevent or reduce *Salmonella* contamination, bacteriophages were isolated from raw chicken offal that would be used for rendering. Bacteriophages were isolated by using the *Salmonella* spp. isolated previously from the finished meals as the host bacteria. For further study, five of the isolated bacteriophages (n = ?) were selected to produce a cocktail for bacteriophage treatment studies. The selection was based on which bacteriophage had the highest lytic activity against 5 pre-determined *Salmonella* serotypes, i.e., Enteritidis, Idikan, Johannesburg, Mbandaka, and Typhimurium. Prior to bacteriophage treatment studies, the optimal bacteriophage cocktail concentration was determined by multiplicity of infection (MOI) optimization. Initial results indicated that an actively growing culture is needed for lytic activity of bacteriophages. When using an actively growing *Salmonella* cocktail, the effectiveness of the bacteriophage cocktail was shown to increase by raising the MOI from 1 to 10, whereas MOI of 50 didn't enhance the lytic activity further. MOI optimization also revealed that resistance strains of the *Salmonella* spp. are selected for quickly (12 h), but that the lytic activity of the

bacteriophage treatment is easily extended through the addition of a different bacteriophage cocktail at the 12 h mark.

The optimized bacteriophage cocktail was able to successfully reduce *Salmonella* levels on all tested environmental surfaces (HDPE plastic, cement, rubber, stainless steel). Treatment of *Salmonella* cells attached to the environmental surfaces resulted in a 2 log CFU/cm^2 reduction at 40° and 30°C, and ca. 1 log CFU/cm^2 reduction at 20°C on all surface materials. The presence of an organic layer on the surface had identical levels of reduction, indicating the organic material does not interfere with the bacteriophage's lytic activity. Treatment of the single species biofilm resulted in ca. 1-2, 2-3, and 1 log CFU/cm^2 reduction in *S.* Enteritidis H4717 populations on all surface materials at 20, 30, and 40°C, respectively, as compared with ca. 0.5, 1.5-2, and 0.5 log CFU/cm^2 reduction in *S.* Enteritidis populations of the double species biofilm under the same experimental condition.

In addition to the surface materials, the bacteriophage cocktail was shown to reduce *Salmonella* levels in raw chicken offal. When treating irradiated raw offal that was artificially contaminated with the *Salmonella* cocktail (10^5 CFU/g), the bacteriophage cocktail reduced *Salmonella* levels by ca. 2.0, 2.7, and 2.5 log CFU/g at 20°, 30°, and 40°C, respectively. The bacteriophage was also capable of reducing *Salmonella* levels to the same degree, i.e., ca. 2.0, 2.2, and 2.2 log CFU/g at 20°, 30°, and 40°C, respectively, in the non-sterile raw chicken offal at the presence of background microorganisms.

The bacteriophage cocktail was also evaluated as a feed additive. The bacteriophage cocktail was lyophilized and added into the animal meals (blood, feather,

and poultry), which were artificially contaminated with a five-strain *Salmonella* cocktail at initial level of ca. 10^5 CFU/g. A series of dehydration studies revealed that the addition of dehydrated bacteriophages to finished meals does not reduce the level of *Salmonella* present upon rehydration; however, there was an observable difference between those samples containing the bacteriophages and those that did not after 12 h of rehydration, with those containing the bacteriophages having lower levels of *Salmonella* (ca. 2 log CFU/g difference ??). Our results also revealed that the bacteriophage cocktail's stability was reduced quickly when applied in a dehydrated form. The concentration of the dehydrated bacteriophage decreased by 1.5 log PFU/g within the rendered meal over a 4 week period at 30°C. However, the stability of the bacteriophage was maintained well when the bacteriophage was added to the animal feed in liquid form and stored under refrigeration conditions (4°C). Under these conditions, the bacteriophage cocktail's concentration decreased by 0.23 log PFU/g over a 4 week period.

The liquid bacteriophage was then supplemented into animal feed and given to mice during an animal trial. The mice that had been given a diet containing the bacteriophage for a period of a week prior to *Salmonella* inoculation were not infected as evidenced through fecal sampling. These mice shed no *Salmonella* in their feces over a 4 week period. Mice that had not been given the diet supplemented with the bacteriophage shed *Salmonella* in their feces for a period of 2.5 weeks. Histological analysis of the liver and intestine also indicated no observable signs of inflammation in those mice given the bacteriophages. In mice not receiving the bacteriophage treatment, venous dilation, cholangiohepatitis, and monocytes in the portal areas were observed in the liver.

Our results indicate that the *Salmonella* contamination of finished rendered meals is likely the source of cross-contamination between the environment or the incoming raw material and the finished products. The risk for cross-contamination may be reduced through the use of bacteriophage treatment which was found to reduce ca. 99 to 99.9% of *Salmonella* levels on both the environmental surfaces and raw material. The bacteriophage cocktail was also found to have the potential to be used as a feed additive to reduce pathogen levels within an animal host when added to the feed in liquid form just prior to consumption.

DEDICATION

I would like to dedicate this work to my wife, Miranda Kinley, and to my precious daughter, Gracyn Kinley. Without their love and support, I would have never made it through this.

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First, I would like to thank Dr. Xiuping Jiang for her constant guidance and support over the past five years. I would like to thank Dr. John M. Henson, Dr. Tzuen-Rong J. Tzeng, and Dr. Paul Dawson for their assistance. In addition, I am eternally grateful to the past and present food microbiology lab members for their assistance, support, and friendship.

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CHAPTER ONE

LITERATURE REVIEW

The Rendering Industry: An Overview

In the U.S. alone, approximately 100 million hogs, 35 million cattle, and eight billion chickens are slaughtered for consumption annually (Meeker and Hamilton, 2006). This leads to a large amount of by-products which include, skin, hair, hides, hoofs, horns, feet, heads, bones, toe nails, blood organs, glands, intestines, muscle and fat tissue, feathers, shells, and in some instances an entire carcass. On average, approximately 50% of an animal used for food production (meat, milk, and eggs) is not consumed by humans. To break it down further, "approximately 49% of the live weight of cattle, 44% of the live weight of pigs, 37% of the live weight of broilers, and 57% of the live weight of most fish species are materials not consumed by humans" (Ockerman and Hansen, 1988; Meeker and Hamilton, 2006). The increasing use of pre-packaged and ready-to-eat (RTE) meat products has further increased the amount of material intake for rendering over the past few years. Currently, the U.S. has an approximate raw material intake of 54 billion pounds per year as compared with ca. 5 billion pounds of raw material in Canada. The composition of these raw materials is reported to be 60% water, 20% protein and mineral, and 20% fat (Meeker and Hamilton, 2006). These materials spoil quickly and are highly contaminated with microorganism including those that are pathogenic to humans; therefore, rendering is a fast and efficient way of disposing of these materials and eliminating the microorganisms from the finished product (Meeker and Hamilton, 2006; Aird and Spragg, 2005).

The rendering process breaks down these animal by-products (those not suitable for human consumption) by physical and chemical manipulation. In general there are four categories of rendering systems: wet rendering, batch rendering, dry continuous, and continuous low-temperature rendering (Ockerman and Hansen, 1988). All rendering methods use heat to

thoroughly cook the material followed by moisture removal and fat extraction. The temperature used and the length of application are crucial to the quality of the finished product. Although both factors vary depending on the raw material being processed, the rendering process involves a set of general steps regardless of the raw material. Initially all raw material is transported to an area in the facility where it is ground and fed onto a conveyer that transports it into the cooker. The cooking process typically lasts for 40-90 minutes at 115° to 145°C (Meeker and Hamilton, 2006). Once the material has been thoroughly cooked, the melted fat is separated and the majority of the moisture is removed. This separation of fat is performed in a closed vessel using a screw press. The remaining material, "cracklings", is ground further and has additional moisture removed (Ockerman and Hansen, 1988). When the "cracklings" have been ground to sufficient size particles, both the processed protein meal and fat are either transferred to a storage facility or shipped out to a consumer (Meeker and Hamilton, 2006).

There are approximately 300 rendering facilities in North America. The rendering facility processes the inedible animal by-products into many valuable products, the largest of which is meals used as supplements in animal feed. The type of meal is dependent on the raw material it is produced from. There is a wide range of meal types which includes meat and bone meal, meat meal, poultry meal, hydrolyzed feather meal, blood meal, and fish meal (Meeker and Hamilton, 2006). A breakdown on the application of two meal types (meat and bone meal and blood meal) is presented in Table 1.1.

Table 1.1 Domestic use of animal proteins by various animal species[1]

	Meat and Bone Meal		Blood Meal	
Species fed	Million pounds	Percentage	Million pounds	Percentage
Ruminants	567.4	10	158.55	70
Swine	737.6	13	45.3	20
Poultry	2439.6	43	22.65	10
Pet food	1304.9	23	-	-
Other	624.1	11	-	-
Total	5673.5	100	226.5	100

1 (Sparks, 2001)

Rendering Processes

Modern rendering facilities have made vast improvements in the technology and processes used over the past several years. Current facilities are constructed so as to separate the incoming raw material from other areas of the facility such as the processing and storage areas. The rendering process is controlled by a computer system that regulates the temperature, time, and in some cases pressure during processing. The technology ensures that the raw material receives sufficient treatment to achieve thermal kill time requirements for the pathogenic microorganisms. In the mean time, it prevents overheating of the material which will lead to a decrease in the nutritional value and digestibility of the finished product (Meeker and Hamilton, 2006).

Essentially there are two major types of rendered processing, wet and dry rendering. In a wet rendering system, there is high moisture content in the finished product, which may or may not be dried. This form of rendering is primarily used for the production of edible fats and oils. Early wet rendering systems were of simple design and involved cooking the fat in an open kettle heated over a wood fire or hot coal. The fat would rise to the top and was skimmed off (Anderson, 2006). Current methods for wet rendering include loading the raw material into a

vertical digestor where steam is injected into the material through the perforated plates in the digestor. This method of rendering is being gradually phased out due to several disadvantages including: long cooking times, labor intensive, and up to 25% of the meal can be lost during processing (Ockerman and Hansen, 1988).

Dry rendering systems are the most common form of rendering used today. Dry rendering is accomplished through one of two system designs, batch rendering or continuous rendering. The use of batch cooking rendering systems began to be universally used during the later part of the twentieth century. A batch system is designed to be "loaded, processed to a percentage dry, and then discharged for fat separation" (Anderson, 2006). In a batch system, a batch cooker can be used to cook, dry, hydrolyze, or process raw material. Originally, large pieces of animal meat or offal would be pressure cooked in a batch system prior to drying, which would make the bones softer and easier to handle. Modern particle reduction technology has eliminated the need for pressure cooking, but it is still used in Europe as means to further reduce bovine spongiform encephalopathy (BSE) causing prions. Pressure cooking is being used in the processing of hair and feathers to make the associated proteins more digestible. For example, the pressure is needed to break down the bonds in the keratin proteins found in feathers. While chemicals could break the bonds post-processing by 100%, the chemicals also destroy the amino acids produced from protein degradation (Anderson, 2006). While batch systems do offer the major advantage of being able to cook, pressurize, and sterilize within the same vesicle, it has more disadvantages than continuous rendering systems. These disadvantages include: the meat based by-products must be cut and washed which adds ca. 35% to the moisture content, problems processing gelatinous material, the material is not contained in enclosed vesicles making it susceptible to contamination, labor intensive, and inefficient drying (Ockerman and Hansen, 1988).

Continuous rendering has undergone many structural changes over time. One of the earliest continuous systems developed was the Anco Strata-Flow system. This system was composed of batch cookers that had been modified and linked together to form a continuous processing system with each batch cooker responsible for a specific aspect of the rendering process. Since then, several adaptations have been made to the design including the DUKE, Carvo-Greenfield, Atlas, and the Stord-Bartz systems (Anderson, 2006). Regardless of the design, the flow of raw material through a continuous rendering system is the same. Initially, raw material is transferred into a grinder where the material is ground into small uniform particles. The particles are then transferred into a continuous cooker where they are heated to 115°-145°C with steam. Once the material has been adequately cooked, the liquid fat and solid material are separated using a drainer conveyor. The solid material is fed into a screw press to reduce the fat content to ca. 10-12%. The solid material is then combined to form the finished meal.(Ockerman and Hansen, 1988; Anderson, 2006). The continuous rendering system is outlined in Figure 1.1 (Anderson, 2006).

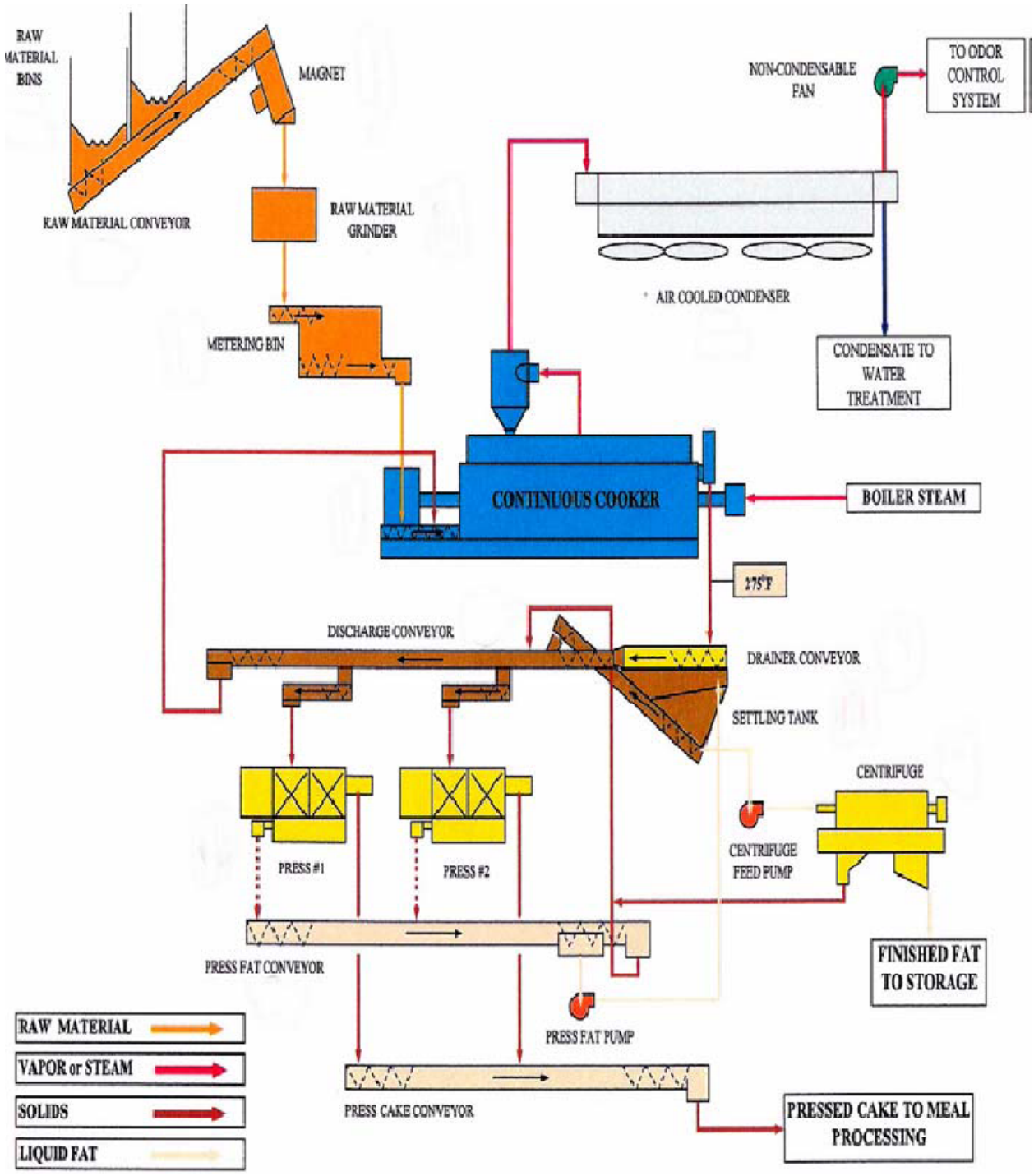

Figure 1.1. Continuous Rendering System Outline[1]

1 Available at: http://nationalrenderers.org/assets/flow_charts.pdf

Finished Rendered Products

Annually, the U.S. rendering industry produces ca. 11.2 billion pounds of protein and 10.9 billion pounds of fats. About 85% of these products are then used in the production of animal feed ingredients (Meeker and Hamilton, 2006). The composition of these feed ingredients is defined by the National Research Council (NRC) or the Association of American Feed Control Officials (AAFCO), which puts out and updated manual annually with feed ingredient guidelines (Aird and Spragg., 2005). Table 1.2 outlines the nutrient composition of commonly used rendered products. As of 2006, the AAFCO manual referenced ca. 125 individual animal by-products produced in the U.S. (Meeker and Hamilton, 2006). Some major meal types are discussed in detail in the following paragraphs.

According to AAFCO, meat and bone meal (MBM) is made from mammalian tissues including the bone but excluding blood, hide, horn, hooves, stomach or rumen contents, or hair. MBM must also have a phosphorus content of at least 4% and a calcium level less than 2.2 times the phosphorus level. If the phosphorus content is less than 4%, the meal is defined as meat meal (Meeker and Hamilton, 2006). In addition, the meal cannot contain more than 12% and 9% indigestible pepsin residue and crude protein, respectively; however, in other countries such as Australia the amount of indigestible pepsin is slightly higher at ca. 15% (Meeker and Hamilton, 2006; Aird and Spragg., 2005). MBM of poultry origin is used for all species of livestock; however, ruminants cannot be fed meals that have been made from ruminant animals as outlined by the feed law passed by the FDA in 1997 (title 21, Code of Federal Regulations, Part 589.2000) to prevent the transference of BSE (Meeker and Hamilton, 2006).

Poultry bone meal (PBM) by definition is composed from the cleaned carcass of poultry including the neck, feet, undeveloped eggs, and intestines. Some feathers are unavoidably

incorporated into the raw material, but in general the product is free of feathers. Like MBM, the calcium level in PBM must not exceed the phosphorus level by more than 2.2 times the amount (Meeker and Hamilton, 20006). The NRC has the calcium content listed at ca. 10.3% and the phosphorus content at 5.1% (Aird and Spragg., 2005). PBM is usually of high quality with essential amino acids, vitamins, minerals, and fatty acids, so it has been commonly used to produce pet grade feed (Meeker and Hamilton, 2006; Aird and Spragg., 2005).

The feathers not used to produce poultry meal can be used to produce feather meal (FeM). The largest market for unused feathers is in meal production (Ockerman and Hansen, 1988). A minimum of 75% of the meal's crude protein must be digestible by pepsin. The undecomposed feathers are cooked under pressure to break the keratin protein bonds, which results in a feather meal that is free-flowing and easily digestible by all species of livestock (Meeker and Hamilton, 2006). Typically the finished feather meal far exceeds the digestibility guidelines set by AAFCO, but digestibility in ruminant animals has been shown to increase through the addition of urea to the meal (Ockerman and Hansen, 1988). A unique aspect of this meal type is that it is an excellent source for sulfur containing amino acids, particularly cystine (Meeker and Hamilton, 2006); however, it is deficient in lysine, methionine, histidine, and tryptophan (Ockerman and Hansen, 1988).

In addition to inedible animal parts and feathers, clean (free of hair, urine, and stomach contents) blood from slaughtered animals can also be used to produce finished, rendered by-products. Initially, blood is treated by removing a large amount of the moisture by mechanical dewatering or turning the blood into a semi-solid state by coagulating it with steam. In this instance, the semi-solid masses are flash dried to rapidly remove the remaining moisture content. According to AAFCO guidelines, the minimum biological activity of lysine within the finished

meal must be at least 80% (Meeker and Hamilton, 2006; Ockerman and Hansen 1988). During the 1960s and 1970s, blood meal production was limited due to the vat-drying procedures which significantly lowered the bioactivity of lysine. With newer methods of drying (ring or flash-drying), blood meal not only has the highest amount of lysine but is the richest source of protein for animal feed as well (Meeker and Hamilton, 2006). The ring drying system has been shown to increase the amount of biologically active lysine by ca. 34% and the amount of protein by 7.5% in blood meal (Ockerman and Hansen, 1988).

Table 1.2 Nutrient composition of animal proteins[1]

Item	Meat and Bone Meal	Blood Meal[2]	Feather Meal	Poultry Meal
Crude Protein %	50.40	88.90	81.00	60.00
Fat %	10.00	1.00	7.00	13.00
Calcium %	10.30	0.40	0.30	3.00
Phosphorus %	5.10	0.30	0.50	1.70
TME_N[3] kcal/kg	2666	3625	3276	3120
Methionine %	0.70	0.60	0.60	1.00
Cystine %	0.70	0.50	4.30	1.00
Lysine %	2.60	7.10	2.30	3.10
Threonin %	1.70	3.20	3.80	2.20
Isoleucine %	1.50	1.00	3.90	2.20
Valine %	2.40	7.30	5.90	2.90
Tryptophan %	0.30	1.30	0.60	0.40
Arginine %	3.30	3.60	5.60	3.90
Histidine %	1.00	3.50	0.90	1.10
Leucine %	3.30	10.50	6.90	4.00
Phenylalanine %	1.80	5.70	3.90	2.30
Tyrosine %	1.20	2.10	2.50	1.70
Glycine %	6.70	4.60	6.10	6.20
Serine %	2.20	4.30	8.50	2.70

1 (National Research Council 2004)

2 Ring or flash dried

3 TME_N = true metabolizable energy nitrogen corrected (Dale, 1997)

Microbiological Safety of the Rendering Process

In addition to producing a profitable end product, the rendering industry also plays a major role in removing pathogenic microorganisms from the food chain. The effective treatment of pathogenic microorganisms in the incoming animal by-products has been documented by some research (Troutt et al., 2001).

The incoming raw material serves as a reservoir for many pathogenic bacteria. Cattle have been reported with a 23% contamination rate with *E. coli* O157:H7 (Smith et al., 2001), a 50% contamination rate with *Salmonella* (Troutt et al., 2001), and a 39% contamination rate with *Cryptosporidium parvum* (Huetink, 2001). Poultry has been reported with up to a 100% contamination rate by *Salmonella* (Council of Agricultural Science and Technology, 1994). Swine have been reported to be contaminated with *Salmonella* (46%) and *Yersina enterocolitica* (49%) (Swanenburg et al., 2001; Council of Agricultural Science and Technology, 1994).

One of the major microbial contaminants in finished feed is *Salmonella*. As mentioned previously, the rendering process should effectively eliminate *Salmonella,* but one of the major issues facing the rendering industry is cross-contamination from the incoming raw material (Ockerman and Hansen, 1988). Once the meal is contaminated with *Salmonella,* it is incorporated into the finished feed given to livestock. This allows *Salmonella* to continuosly enter the food chain. Several studies have been able to link cases of *Salmonella* infection in humans to contaminated animal feed. Boyer et al. (1958) established a link between serotypes of *Salmonella* recovered from humans and animals to feed ingredients and animal feed. A year later, Watkins et al. (1959) was able to recover 28 different serotypes of *Salmonella* from 18.5% of animal by-products sampled. Pomeroy et al. (1961) expanded on Watkins work by collecting

samples from 22 states across the United States. In total, *Salmonella* was identified in 170 of the 980 samples tested with 43 serotypes identified.

The problem with *Salmonella* was further illustrated by a *Salmonella agona* outbreak. From 1969 to 1970, *S. agona* was identified as a public health concern in the United States, United Kingdom, Netherlands, and Israel. In each country, initial detection of *S. agona* in fish meal was followed by the detection of the pathogen in domestic animals and humans (Clark et al. 1973). By 1972, *S. agona* was the 8th most commonly isolated serotype in the United States. During a period from March to May of that year, an outbreak of *S. agona* occurred in Paragould, Arkansas. In total, seventeen people were infected. The source was traced back to a local restaurant which had received poultry products from a Mississippi farm that had used fish meal contaminated with *S. agona* (Franco, 2006).

From 1965 to 1970, the animal health division of the United States Department of Agriculture (USDA) collaborated with the Federal Drug Administration (FDA) to sample animal by-products and finished meals to determine levels of *Salmonella* contamination. In response to some of the initial findings, Commissioner Goddard made the decision within the FDA to include food produced for animal consumption as part of Section 201 (f) of the Federal Food, Drug and Cosmetic Act in 1967. This meant that *Salmonella* contamination in animal feed was now considered an adulterant by definition of the Act (Franco, 1999).

While studies have indicated that *Salmonella* can be detected in finished animal by-products, the amount present after processing declines rapidly. Sutton et al. (1992) demonstrated that in a sample of MBM stored at 28.8°C, *Salmonella* levels decrease from 30 CFU/g to below the detection limit within 2 days of storage. Even when *Salmonella* is present, animal by-products still pose less of a risk for *Salmonella* contamination when compared to meals

composed of vegetable proteins. Hamilton (2002) determined the incidence rate of *Salmonella* in animal by-product meals was higher in the Netherlands, Canada, and the U.S., while the levels were higher in the vegetable proteins in Germany and the UK. The highest incidence rates for both proteins were observed in products produced in the U.S. While the animal proteins had a higher range of incident rates, the risk factor associated with vegetable proteins is higher due to the fact that makes up a larger percentage of the finished feed. The animal proteins have a risk factor of 0.9-1.68 and vegetable proteins have a risk factor of 1.743-8.964 for *Salmonella* (Hamilton, 2002)

The establishment of linkages between human disease and contaminated animal by-products along with the FDA's incorporation of animal feed into the definition of food led to the rendering industry began placing a higher emphasis on the biological safety of the products they produce. In 1984, the rendering industry founded the Animal Protein Producers Industry (APPI) to regulate biosecurity within the industry, such as *Salmonella* screening or developing protocols such as hazard analysis and critical control plans (HACCP) to ensure the safety of the product, and to continuously educate the industry on new issues that may impact their products (Franco, 2006).

Since the formation of APPI, vast improvements have been made to the rendering process and more recent studies have provided data that indicates finished meals are free of pathogenic bacteria including *Salmonella* as it leaves the cooker. Troutt et al. (2001) tested raw products used in production at 17 rendering facilities in the mid-western United States. The results showed there were high levels of *Salmonella* spp., *Listeria monocytogenes, Campylobacter jejuni,* and *Clostridium perfringens* contamination. When the processed meals were tested, none of these pathogens were detected (Table 1.3). In addition to bacteria, viruses are also effectively

eliminated during the rendering process. The time and temperature used by the rendering industry was always thought to be sufficient to kill viruses within the by-products. Pirtle (1999) demonstrated this using pseudorabies virus (PRV, a common viral disease in pigs) as a model microorganism. To determine the effectiveness of the rendering process, raw materials with varying degrees of viral contamination were processed and the resulting MBM was analyzed for the presence of the virus. The results indicated that the virus was unable to survive the rendering process (Pirtle, 1999).

Table 1.3 Pathogen elimination from finished rendered products

Pathogen	Raw Tissue (% positive)	Finished Product (% positive)
Clostridium perfringens	71.4	0
Listeria spp.	76.2	0
Listeria monocytogenes	8.3	0
Campylobacter spp.	29.8	0
Campylobacter jejuni	20.0	0
Salmonella spp.	84.5	0

Troutt et al. (2001)

Two years after the establishment of the APPI (1986) BSE was confirmed in the United Kingdom and believed to have originated from MBM produced from sheep by-products (Kimberlin, 1990). This led to the USDA conducting an 8 year study in an attempt to demonstrate that the rendering process kills the prions responsible for BSE and they are not transmitted orally to cattle via consumption of finished MBM. Neonatal calves were fed a diet of raw brain or finished MBM from sheep infected with scrapie. During the eight year period, the calves were checked for signs of disease, lesions, or prion protein deposits associated with scrapie or BSE (Cutlip et al., 2001). The results showed that experimental calves given the MBM diet did not exhibit any clinical signs or develop lesions associated with BSE. In addition, examination of spinal cord and brain samples did not indicate the presence of any prions (Cutlip

et al., 2001). Howevere, a disease found in humans, Creutzfeldt-Jakob disease, linked to the consumption of cattle suffering from BSE was identified a decade after the intial discovery of BSE. This led to the FDA's decision to ban the use of feed produced from ruminants as a feed ingredient for ruminant animals (Franco, 2006).

While there are some risks associated with finished animal by-products, the rendering process is still the best means for the disposal of the raw by-products and mortalities. The rendering industry provides numerous advantages including: infrastructure, volume reduction, controlled processing, established regulations, and timely processing (Hamilton et al., 2006). To validate this claim, the United Kingdom Department of Health (2001) conducted a study evaluating the risks involved with various methods of disposal for animal by-products. Their study determined biological hazards were negligible if the material was treated by rendering, incineration, or funeral pyre. While biological hazards were eliminated, incineration and pyres create chemical hazards associated with burning. The rendering process was the only means of disposal that successfully eliminated the biological and chemical hazards. The only exception was BSE, which was found to be a negligible risk to humans if the solid material was incinerated (Hamilton et al., 2006). A summary of the study can be seen in Table 1.4.

Table 1.4 Potential health risks of various methods of animal by-products disposal[1]

	Disposal Options				
Disease/Hazardous Agent	Rendering	Incineration	Landfill	Pyre	Burial
Campylobacter, E. coli, Listeria, Salmonella, Bacillus anthracis, C. botulinum, Leptospira, Mycobacterium tuberculosis var bovis, Yersinia	very small	very small	moderate	very small	high
Cryptosporidium, Giardia	very small	very small	moderate	very small	high
Clostridium tetani	very small	very small	moderate	very small	high
Prions for BSE, scrapie	moderate	very small	moderate	moderate	high
Methane, CO_2	very small	very small	moderate	very small	high
Fuel-specific chemicals, metal salts	very small	very small	very small	High	very small
Particulates, SO_2, NO_2, nitrous particles	very small	moderate	very small	high	very small
PAHs, dioxins	very small	moderate	very small	high	very small
Disinfectants, detergents	very small	very small	moderate	moderate	high
Hydrogen sulfide	very small	very small	moderate	very small	high
Radiation	very small	moderate	very small	moderate	moderate

1 (U.K. Department of Health 2001)

In addition to potential biological and/or chemical hazards, the other mean of disposal can simply not handle the volume of raw materials. When the by-products are disposed of in landfills, an additive such as saw dust is mixed in to reduce high moisture content in raw materials. The addition of saw dust can raise the volume of material by 25% to a point that disposing of all the animal by-product and mortalities generated in 1 year would consume of

25% of the total landfill space in the United States (Sparks, 2001). Another alternative for disposal of animal by- products is composting, but composting is limited to small scale production due to the amount of material need to balance the carbon and nitrogen ratio and moisture content in the raw by-products and mortalities (Hamilton et al., 2006). Based on the pork industry alone, a space of ca. one trillion cubic feet would be needed to compost the amount of material rendered each year (Glanville, 2001). While effective at eliminating biological hazards, incineration is also limited by the volume of raw by-products produced each year. The volume of raw material would require large amounts of fossil fuel which is also cost prohibitive. In addition, the ash produced from the raw by-products would have to be disposed of as well (Hamilton et al., 2006).

This literature review will focus mainly on the microbiological safety of the rendered products. Two microbial species, *Enterococcus* and *Salmonella*, will be discussed in detail in the following sections.

Enterococci: General Overview

Enterococci are ubiquitous and found in most environments, but most commonly they can be isolated from an animal's alimentary tract, the soil, water, and food. They have acquired many attributes that ensures their survival in such diverse environments including heat tolerance up to 45°C (higher in certain species), tolerance of high salt concentrations and a broad range of pH levels. In addition to these characteristics, the enterococci are commonly multi-drug resistant which has allowed them to persist with in an animal host (Facklam et al., 2002). The common presence within the alimentary tract of livestock and its ability to survive in various environments makes enterococci a concern for finished rendered meals. Like *Salmonella*, there is an opportunity for enterococci contamination of rendered animal meals due to cross-

contamination with the incoming raw material (Ockerman and Hansen, 1988). Once in the feed, enterococci can enter the food chain. While most strains of enterococci lead to treatable or self-limiting diseases, there is a major concern for vancomycin resistant enterococi (VRE), which will be discussed in more detail later.

Thiercelin (1899) first reported what is now thought to be enterococci in human fecal samples. He described the bacterium as appearing in pairs or short chains. Andrews and Horder (1906) identified a bacterium of fecal origin, *Streptococcus faecalis*, which would clot milk. Thirteen years later. Orla-Jensen (1919) identified an organism with different fermentation patterns, which was *Streptococcus faecium.* A third bacterium, *Streptococcus durans*, of fecal origin was discovered by Sherman and Wing (1935, 1937) with less fermentation activity. In 1967, a new species *Streptococcus avium* was added to the group by Nowlan and Deibel (1967). Kalina (1970) proposed a new genus name, *Enterococcus*, should be established. Genetic evidence provided by Schleiter and Kilpper-Balz (1984) showed that *S. faecalis* and *S. faecium* were significantly different from other member of the *Streptococcus* genus resulting in the formation of the new genus, *Enterococcus.*

Disease, Virulence, and Antibiotic Resistance of Enterococci

While enterococci do inhabit the gastrointestinal tract of the majority of humans, they are still responsible for a wide variety of diseases. Enterococci have been linked to endocarditis (MacCallum et al., 1899; Rantz and Kirby, 1943; Megran, 1992), respiratory tract infections (Rantz and Kirby, 1943), and urinary tract infections (Braude, 1973; Gross et al., 1976; Richards et al., 2000; Rantz and Kirby, 1943; Rantz, 1942). The most common species associated with human illnesses are *E. faecalis* and *E. faecium*; however, the following species have also been linked to human disease: *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. raffinosus*

(Murray, 1990). Recently, enterococci have been identified as the leading cause of surgical site infections (17.1%), second leading cause of blood stream infections (11.5%), and the third leading cause of nosocomial urinary tract infections (14.3%) (Richards, 2000). In particular, *E. faecalis* accounts for up to 80% of the nosocomial infections resulting from enterococci (Huycke et al., 1998; Sahm, 2000).

The high incidence of enterococci infections can be partially attributed to a large set of virulence factors. For example, *E. faecalis* is capable of producing the enzymes cytolysin, gelatinase, and serine protease which play a role in host tissue degradation (Hancock and Gilmore, 2000; Dupont et al., 1998; Burns et al., 1996; Okamoto et al., 1997; Plaut, 1983; Prokesova et al., 1992; Schultz and Miller, 1974; Sundqvist et al., 1985; Lantz et al., 1991; Travis et al., 1994). The *Enterococcus* genus is also capable of producing an aggregation substance (Clewell, 1993) and Enterococcal surface protein (Esp) (Shankar et al., 2001) which aid in conjugation and adherence to epithelial cells, respectively. Enterococci may also be surrounded by a capsule allowing them to evade phagocytosis (Gilmore, 2002). Enterococccci also produce large amounts of superoxide. The superoxide fragments the chromosome of epithelial cells in the colon granting the enterococci entrance into the bloodstream (Huycke et al., 1996; Huycke et al., 2001).

In addition to the treatment of human infections, antibiotics have other applications such as animal growth promoters. There are four primary applications for antibiotics in food animals. The first is the therapeutic treatment of infections within the animal. The second use is for the treatment of a healthy animal living in a flock where at least one animal is infected in an effort to prevent infection. A third use is as a prophylactic way to treat animals to prevent the occurrence

of disease. The final means is as a growth promoter, which prevents the animal from having to fight off infection and the growth of the animal will improve (Aarestrup, 2002).

The Union of Concerned Scientists estimated that in the United States 24.6 million pounds of antimicrobial agents are used on feed animals every year compared to the 3 million pounds used for human treatment (Mellon et al., 2001). Similarly, the European Agency for Evaluation of Medical products reported that in 1997 that ca. 10 million pounds of antimicrobial agents were used for feed animals across Europe (EMEA, 1999).

While enterococcal infections are rarely treated in animals, enterococci are persistently present within the animal's intestinal tract. These enterococci are then constantly exposed to an antimicrobial agent when it is being used as a growth promoter or prophylactic, which could lead to the selection of strains resistant to the antimicrobial agent used (Aarestrup, 2002). Starr and Reynolds (1951) demonstrated this possibility with coliforms in the intestinal tracts of turkeys being continually fed feed supplemented with streptomycin. Their results indicated the presence of streptomycin resistant coliforms. Two other studies also demonstrated that the use of tetracycline as a growth promoter selected for resistant strains of group D streptococci, enterococci, within chickens (Barnes, 1958; Elliot and Barnes, 1959).

Due to the overuse of antibiotics as growth promoters, enterococci have developed high levels of resistance to various classes of antibiotics including aminoglycosides, chloramphenicol, glycopeptides, macrolides, streptogramin, and tetracycline. Several studies have idenitified enterococci resistant to one or more of these classes (Table 1.5).

The antimicrobial agents used for therapy and growth promotion in these feed animals are essentially identical to the classes of antibiotics used for human treatment (Aarestrup, 2002). This causes concern that the antimicrobial agents used in growth promotion can results in a

decrease in efficiency of those used for human treatment. This concern was first acted upon in 1969 by the Swann committee (Swann, 1969). The committee developed a report recommending that any antibiotic used as a method of treatment not be used for growth promotion. This recommendation was partially adopted by the European Union, but the United States continued using antibiotics such as penicillin in large amounts (Aarestrup, 2002). The antibiotic avoparcin, which is in the glycopeptide class, was banned from use as a growth promoter in Denmark in 1995 due to concerns that it would select for enterococci resistant to vancomycin and that these resistant strains could then spread to the human population. This led to a complete ban of avoparcin in all countries within the European Union in 1997. A year later, virginiamycin was banned due to cross resistance with quinupristin/dalfopristin. The antibiotics bacitracin, spiramycin, and tylosin were also banned as growth promoters in 1998 by the European Union (Aarestrup, 2002).

Table 1.5 Reported cases of antibiotic resistant enterococci in food animals[1]

Class	Antibiotic	*Enterococcus spp.*	Summary	Ref.
Aminoglycosides	Gentamicin	*faecalis*	22% chicken meat samples	Thal et al., 1995
	Gentamicin	*faecalis*	11.5% broiler	Yoshimura et al., 2000
	Kanamycin	*faecalis* and *faecium*	30% bovine mastitis	Jayorao and Oliver, 1992
	Kanamycin	*faecalis*	62% chickens and 36% swine	Rollins et al., 1985
	Streptomycin	*faecalis* and *faecium*	80% bovine mastitis	Jayorao and Oliver, 1992
	Streptomycin	*faecalis*	64% chickens and 57% swine	Rollins et al., 1985
	Streptomycin	*faecium* and *faecalis*	14.4% and 67.9% broilers	Yoshimura et al., 2000
Chloramphenicol	Chloramphenicol	*faecium*	1% broilers and 7% swine	Aarestrup et al., 2000a
	Chloramphenicol	*faecalis*	2% broilers and 4% swine	Aarestrup et al., 2000a
	Chloramphenicol	*faecium* and *faecalis*	40% raw meat samples	Pavia et al., 2000
	Chloramphenicol	*faecalis*	2.6% broilers	Yoshimura et al., 2000
Macrolide	Erythromycin	*faecium*	12.7% broilers and 46.7% swine	Aarestrup et al., 2001
	Erythromycin	*faecalis*	28.1% swine	Aarestrup et al., 2001
	Erythromycin	*faecium* and *faecalis*	65.3% and 89.7% broilers	Yoshimura et al., 2000
	Tylosin	*faecalis*	50% poultry	Dutta and Devriese, 1982
	Tylosin	*faecium*	67% poultry	Dutta and Devriese, 1982
	Tylosin	*faecium* and *faecalis*	65.4% and 89.7% broilers	Yoshimura et al., 2000
	Tylosin	*faecium*	75.4% broilers and 81.8% swine	Aarestrup et al., 2000b
	Tylosin	*faecium*	82% broilers and swine	Butaye et al., 2001
	Tylosin	*faecalis*	54% broilers and 86% swine	Butaye et al., 2001
Streptogramin	Quinupristin/dalfopristin	*faecium*	79% broilers and 60% swine	Aarestrup et al., 2000a
	Quinupristin/dalfopristin	*faecalis*	90% broilers and 98% swine	Aarestrup et al., 2000a
	Quinupristin/dalfopristin	*faecium*	51.2% poultry cloacal swabs	Hayes et al., 2001
	Quinupristin/dalfopristin	*faecium*	93% poultry carcass	McDonald et al., 2001
	Quinupristin/dalfopristin	*faecium*	23% 24 day old turkey	Welton et al., 1998
	Quinupristin/dalfopristin	*faecium*	100% 130 day old turkey	Welton et al., 1998
	Virginiamycin	*faecium*	33.9% broilers and 22.5% swine	Aarestrup et al., 2001

	Virginiamycin	*faecium*	75% broilers and 85% swine	Aarestrup et al., 2000a
	Virginiamycin	*faecalis*	95% broilers and 99% swine	Aarestrup et al., 2000a
Tetracycline	Tetracycline	*faecium*	20% broilers and 70% swine	Aarestrup et al., 1998
	Tetracycline	*faecium*	32% broilers and 63% swine	Aarestrup et al., 2000a
	Tetracycline	*faecalis*	59% broilers and 68% swine	Aarestrup et al., 2000a
	Tetracycline	*faecium* and *faecalis*	94.8% and 93.6% broilers	Yoshimura et al., 2000
	Oxytetracycline	*faecium*	92% broilers and 97% swine	Butaye et al., 2001
	Oxytetracycline	*faecalis*	79% broilers and 100% swine	Butaye et al, 2001

1 (Gilmore, 2002)

Vancomycin Resistant Enterococci

Enterococci have also developed high levels of resistance to the glycopeptides. Resistance was first detected in *E. faecium* clinical isolates from France in 1986 (Leclerq et al., 1988) and the United Kingdom (Utley et al., 1989). The first detection of glycopeptide resistant enterococci (GRE) outside of a clinical setting was the occurrence of the vanA phenotype of vancomycin resistance in the sewage and among chickens in the United Kingdom (Bates et al., 1993). As mentioned previously the use of the growth promoter avoparcin was identified as the cause of the spread of GRE. Fecal samples collected from farms using avoparcin as a growth promoter contained high levels of GRE; whereas, samples from farms not using avoparcin were only rarely contaminated with GRE (Aarestrup, 1995; Bayer et al., 1997; Klare et al., 1995). The presence of GRE in food animals has become widespread in Europe (Gilmore, 2002). Aarestrup (1998a) detected glycopetide resistance in 70% and 20% of the *E. faecium* isolates collected from broilers and pigs, respectively, in Denmark. Reports from the Netherlands have indicated that 34% and 80% of the enterococci isolated from the feces of pigs and broilers, respectively, are GRE (Van den Bogaard et al., 1997). While the presence of GRE in Europe has been well documented, there have been no reports of GRE in the US, where avoparcin is not used (Coque et al., 1996).

Vancomycin is a glycopeptide antibiotic that is used to treat infections resulting from antibiotic resistant gram positive bacteria. The first reported cases of vancomycin resistant enterococci (VRE) were in *E. faecium* isolates collected from patients with leukemia in France and Patients in England suffering from renal failure (Leclerq et al., 1988; Uttley et al., 1988). The occurrence of VRE is not limited to Europe. Fridkin and Gaynes (1999) reported that over

20% of enterococci isolates collected in 1998 in the US were VRE. In some hospitals, *E. faecium* has been reported as resistant to vancomycin over 90% of the time (Kak and Chow, 2002).

The emergence of VRE has been attributed to the widespread use of the antibiotic in clinical settings and the use of glycopeptides as growth promoters in animal feed. The variety in VRE genotypes and their sequence variability implies that they did not emerge as a single point mutation (Kak and Chow, 2002). The guanine (G) & cytosine (C) content exhibited in *vanA* and *vanB* type VRE exceeds that typically found in either *E. faecalis* or *E. faecium*, implying that VRE probably acquired gene cluster from other microorganisms (Patel,1999). The proposed theory of evolution is that the gene cluster responsible for resistance was transferred from vancomycin producing organisms to a specific organism with a similar G & C content. These organisms then transferred the gene clusters to enterococci. Once enterococci had gained resistance, the occurence of resistance grew due to the abundant use of glycopeptides. In the US, the problem is more concentrated in clinical settings. The occurrence of VRE in Europe is mostly concentrated in non-human hosts and within healthy humans. The reasons for these patterns can be contributed to the difference in primary use of glycopeptides between the two regions. In the US, glycopeptides have primarily been used as human therapy while in Europe they have been largely used as growth promoters in feed animals (Kak and Chow, 2002).

Vancomycin works by inhibiting the synthesis of the cell wall within gram positive bacteria. Specifically, the glycopeptides binds to the D-alanine-D-alanine terminus of the pentapeptide side chains within the cell wall. Once bound, the drug prevents the formation of hydrogen bonds that bind the peptide side chains to each other. This prevents the cross-linking of the peptidoglycan chains leading to a loss in the structural integrity of the cell wall resulting in cell death (Arthur and Courvalin, 1993; Reynolds, 1989; Walsh et al., 1996).

Resistance to vancomycin in enterococci results from a modification in the composition of the pentapeptide side chains. The D-alanine-D-alanine terminus is replaced by a terminus composed of either D-alanine-D-lactate or D-alanine-D-serine. The substitution with D-lactate decreases the results in a 1000x decrease in the affinity for vancomycin due to the loss of a hydrogen bond (Bugg et al., 1991). In contrast, the substitution with D-serine only results in a 7x decrease in affinity for vancomycin (Billot-Klein et al., 1994).

Six gene clusters have been identified as mediators of vancomycin resistance in enterococci (Table 1.6). Five have been identified as acquired traits, while one gene (*vanC*) is intrinsic. The mechanisms for resistance are genotypically and phenotypically distinct for each gene cluster involving a complex group of enzymes. These enzymes are responsible for detecting the presence of vancomycin, initiating the change in peptidoglycan side chain production to a resistant state, and to eliminate the normal (sensitive) peptide-side chain precursors (Kak and Chow, 2002).

Table 1.6 VRE Genotypes[1]

Genotype	Vancomycin MIC (µg/ml)	Teicoplanin MIC	Expression	Precursor	Species
vanA	64-1000	16-512	inducible	D-Ala-D-Lac	*E. faecalis, E. faecium,* and 7 others
vanB	4-1000	≤1	inducible	D-Ala-D-Lac	*E. faecalis* and *E. faecium*
vanC	2-32	≤1	constitutive inducible	D-Ala-D-Ser	*E. casseliflavus, E. gallinarum,* and *E. flavescens*
vanD	64-128	4-64	constitutive	D-Ala-D-Lac	*E. faecium*
vanE	16	0.5	inducible	D-Ala-D-Ser	*E. faecalis*
vanG	<16	<0.5	?	?	*E. faecalis*

1 (Gholizadeh and Courvalin 2000)

The *vanA* cluster is characterized by high levels of induced resistance to vancomycin (≥64 µg/ml) and teicoplanin (≥16 µg/ml). Typically, enterococci acquire this gene cluster through the incorporation of the Tn1546 transposon or from a Tn3 transposon (Arthur, 1993a; Brisson-Noel et al., 1990). Like the *vanA* cluster, the *vanB* cluster also confers high levels of resistance to vancomycin, but *vanB* does not confer resistance to teicoplanin (Evers et al., 1994; Evers et al., 1993; Quintilions et al., 1993; Williamson et al., 1989). While *vanB* is typically present within the chromosome, it can also be carried on plasmids (Rice et al., 1998). Studies by Quintilioni et al. (1996; 1994) have demonstrated that the *vanB* gene cluster can be transferred between enterococci via the Tn1547 transposon. In addition, Carlos et al. (1998) demonstrated that the *vanB* gene cluster was transmitted simultaneously as induced ampicillin resistance via the Tn5382 transposon. The *vanC* gene cluster is only found within the motile species of enterococci and confers low levels of resistance to vancomycin. The specific genes encoding vanC type resistance are dependent upon the bacterial species. It is encoded for by the *vanC-1* gene in *E. gallinarum*, *vanC-2* in *E. casseliflavus,* and *vanC-3* in *E. flavescens* (Clark et al., 1998; Dutka-Malen et al., 1992; Leclerq et al., 1992; Navarro and Courvalin, 1994). The *vanD* gene cluster confers intermediate levels of resistance to both vancomycin and teicoplanin. Unlike the previous gene clusters, *vanD* is located on the chromosome and is non-transferable (Casadewell and Courvalin, 1999; Perichon et al., 1997). Both the *vanE* and *vanG* confer low levels of vancomycin resistance, can be transmitted, and are inducible (Fines et al., 1999; McKessar et al., 2000).

The *vanA* operon is composed of seven genes (Table 1.7). The first set of genes (*vanH, vanA,* and *vanX*) is directly involved in glycopeptides resistance. The second set (*vanR* and *vanS*) regulate resistance, while the *vanY* gene is responsible for the removal of normal cell wall

precursors. The function of the seventh gene, *vanZ*, is still unclear (Kak and Chow, 2002). Resistance is initiated by the transmembrane segment of *vanS* (protein kinase) which is responsible for sensing the presence of glycopeptides. After sensing the glycopeptides, *vanS* undergoes autophosphorylation which results in the transfer of a phosphoryl group to *vanR*, the response regulator. The activated *vanR* then activates *vanH* by binding to its promoter region. It also bind to its own promoter region leading to further transcription of both the *vanR* and *vanS* genes (Arthur, 1992b). The vanH protein then begins the process of D-Ala-D-Lac production by reducing pyruvate to D-Lac (Arthur, 1992a). The ligase, vanA, then bind the D-Lac to a D-Ala. The newly formed D-Ala-D-Lac are then bound to UDP-N-acetyl muramide to be incorporated into the peptidoglycan layer. The D-Ala-D-Ala components of the tetrapeptide side chains are then removed by vanX, a dipeptidase (Reynolds et al., 1994). VanY finishes the process by removing any remaining D-Ala-D-Ala precursors that were missed by vanX (Arthur et al., 1998).

The *vanB* operon has similar components that function the same way as in *vanA*. The exceptions is that *vanB* contains a *vanW* gene instead of a *vanZ* gene, but like *vanZ* its function is unknown (Quintiliani et al., 1993). The *vanD* operon differs in the fact that the vanY carboxypeptidase is sensitive to penicillin and the vanX dipeptidase has little function (Kak and Chow, 2002). *VanE* operons produce D-Ala-D-Ser subunits rather than D-Ala-D-Lac like *vanA* (Fines et al., 1999; Aries et al., 1999). The less studied *vanG* operon is composed of 7 open reading frames which are believed to lead to the production of D-Ala-D-Ser subunits in a matter similar to *vanC* and *vanE* (McKessor et al., 2000)

Table 1.7 *vanA* Operon Genes and their Functions[1]

Gene	Protein	Function
vanH	dehydrogenase	converts pyruvate to lactate
vanA	Ligase	ligates alanine to lactate to form D-Ala-D-Lac
vanX	Dipeptidase	breaks the D-Ala-D-Ala dipeptide that may have formed
vanY	carboxypeptidase	hydrolysis of any D-Ala-D-Ala pentapeptide precursor
vanR	response regulator	activates transcription of the resistance pathway
vanS	protein kinase	senses presence of glycopeptides and phosphorylates VanR

1 (Gholizadeh and Courvalin 2000)

Enterococci Non-Human Reservoirs and Occurrence in Rendered Products

Although, enterococci are found in numerous environments, patterns in the distribution of a particular species do exist (Aarestrup, 2002). Winslow and Palmer (1910) studied the distribution of streptococci, including enterococci, in humans, horses, and cows in an effort to determine the source of fecal contamination in public water supply. While there were certain species found within all three hosts, the most commonly isolated species from the farm animals were *E. faecalis, E. faecium, E. hirae, and E. durans.* Other less common enterococcal species are typically found in a distinct host species, e.g. *Enterococcus columbae* from pigeons. The colonization of *Enterococcus* within chickens has a distinct age dependent pattern. Younger chicks are mostly colonized by *E. faecalis,* but as the chicks age the *E. faecalis* is rapidly replaced by *E. faecium, E. hirae, E. cecorum,* and *E. durans* (Devriese et al., 1987, 1991; Kaukus et sl., 1987). Similar colonization patterns by species have been reported in cattle as well. Pre ruminant calves typically contain *E. faecalis, E. faecium,* and *E. avium,* which are gradually replaced by *E. cecorum.* The overall *Enterococcus* population level diminishes as the cattle age, and typically, the feces of adult cattle show little to no presence of enterococcal species (Devriese et al., 1992).

Enterococci are a common contaminant of animals used to produce rendered animal meals (beef, poultry, and pork) (Franz et al., 2003). For example, Knudtson and Hartman (1993)

detected levels of 4-8 log CFU/cm^3 on pig carcasses, with *E.faecalis* and *E. faecium* as the dominant species. While there has been little work in the detection of enterococci in finished rendered animal meal, the common occurrence in the animals used as raw material and enterococci's elevated heat tolerance could potentially result in contamanitation of the finished meal. Cullen et al. (2003) and Schwalbe et al. (1999) both demonstrated that enterococi can be isolated from finished rendered meals. In 2003. The FDA conducted a national survey, and enterococci was identified in 84% of the meal samples collected with the highest level of incidence in MBM and *E. faecium* being the dominant species (Cullen et al., 2003).

***Salmonella*: Overview**

Salmonella spp. are gram-negative, bacillus shaped, facultative anaerobic, and motile members of the Enterobacteriaceae family. A list of other biochemical characteristics can be seen in Table 1.8. Typically, *Salmonella* are identified by the fermentation of glucose into gas and acid on triple sugar iron (TSI) medium and will not utilize sucrose or lactose in differential media (Andrews et al., 1994; D'Aoust and Purvis, 1998). While typical *Salmonella* do not utilize sucrose or lactose, Le Minor et al. (1974; 1973) have demonstrated in some cases sucrose and lactose fermentation can be mediated by plasmids.

The nomenclature has changed several times during the 20th century due to biochemical and serological characteristics as well as DNA homology. Advances in the detection of the somatic (O) and flagellar (H) antigens allowed the genus to be split into different "groups" (Le Minor, 1981). This antigenic means of classification was first proposed by White in 1926. White's system of classification was further expanded upon by Kauffman in 1941 giving the modern day Kauffman-White classification system which currently includes over 2,541 different

serovars of *Salmonella* (Popoff et al., 2004). A complete detailing of the changes in nomenclature can be seen in Table 1.9.

Table 1.8 Biochemical characteristics of *Salmonella spp.*[1]

	Biochemical Test									
	Glucose	Lactose	Sucrose	Oxidase	Catalase	Citrate	H_2S	Lysine	Ornithine	Urea
Result	+	-	-	-	+	+	+	+	+	-

1 (D'Aoust and Maurer, 2007)

Table1.9 Taxonomy of *Salmonella*[1]

Diagnostic basis	Features	Serovar Nomenclature	Reference
Biochemical	Five subgenera (1-V)	*S. typhimurium*	(Kauffman, 1966)
Biochemical	Three species (*S. typhi, S. choleraesuis, S. enteritidis*) *Arizona* is separate genus	*S. enteritidis* serovar Typhimurium	(Ewing, 1972)
DNA homology	Single species (*S. choleraesuis*) Seven subspecies (choleraesuis, salamae, arizonae, diarizonae, houtenae, bongori,indica)	*S. choleraesuis* subsp. *Choleraesuis* serovar Typhimurium	(Le Minor et al., 1986)
DNA homology	Single species (*S. enterica*) Seven subspecies (choleraesuis, salamae, arizonae, diarizonae, houtenae, bongori,indica)	*S. enterica* subsp. *Enterica* serovar Typhmurium or *Salmonella* serovar Typhimurium	(Le Minor and Popoff, 1987)
Multilocus enzyme electrophoresis	Two species (*S. enterica* [six subspecies] and *S. bongori*)	*S. enterica* subsp. *Enterica* (I)	(Reeves et al., 1989)

1 (D'Aoust and Maurer, 2007)

Salmonella Occurrence and Presence in Rendered Products

Salmonella continues to be a prominent foodborne pathogen in the human food chain due to its widespread distribution in the natural environment, within the meat (beef, poultry, fish, and shellfish) industry, and the recycling of inedible animal parts (offal) into animal feed (D'Aoust, 1989; D'Aoust 1994). To address this issue, the Food Safety Inspection Service (FSIS) with the USDA published the "Final Rule on Pathogen Reduction and Hazard Analysis and Critical Control Point (HAACP) Systems" in 1996. This document required the meat industries to implement HAACP plans and to take regular samplings to detect the indicator organism *Escherichia coli* biotype I, while the FSIS collected regular samples to determine if the implemented HAACP plans were sufficiently reducing *Salmonella* levels. Prior to the release of the "Final Rule", data indicated that broiler chickens were contaminated with *Salmonella* ca. 24% of the time. In 1999, broiler *Salmonella* contamination levels had been reduced to ca. 11%; however, the rate has gradually increased since 1999 reaching ca. 16% in 2005 (D'Aoust and Maurer, 2007).

Aside from the raw materials, it is possible for the environmental surfaces to contaminate the final products upon contact. Stainless steel, cement, rubber, and high density polyethylene plastic are materials found inside a rendering facility. Kusumaningrum et al. (2003) has demonstrated that *Salmonella* is capable of adhering to stainless steel. While low inoculum levels of *S.* Enteritidis (10 CFU/cm^2) were undetectable on the steel after 1 h. A high inoculum of Enteritidis (10^5 CFU/cm^2) was detected after 96 h. that the presence of *Salmonella* on rubber conveyor belts was documented on a Danish pig farm. A rubber conveyor belt was used to transport the manure from the piggery to an on-site manure dump. *S.* Typhimurium isolates collected from this conveyor belt were identical to Typhimurium isolates that were detected 20

months earlier during the first isolation (Sandvang et al., 2000). It is possible that this strain has been persistent on the conveyor belt and is continuously passed between the manure dump and piggery. The presence of food residues increased the survival time of the bacteria. *S.* Enteritidis can be transferred from the steel to food (Kusumaningrum et al., 2003). The study tested the bacterial transfer to cucumber slices and chicken filets by steel immediately after contamination or 15 minutes after contamination. Directly after contamination, contamination rates were as high as 105 ± 28% for cucumber slices and 94 ± 42% for chicken filets. When the contamination was allowed to sit for 15 minutes, the transfer rates were reduced to 90 ± 27% for cucumber slices and 55 ± 21% for the chicken filets.

Salmonella spp. are capable of producing biofilm on environmental surfaces (Jones and Bradshaw, 1996). *Salmonella* Weltevreden formed biofilms on high density polyethylene plastic, cement, and stainless steel with the following cell densities: 3.4×10^7, 1.57×10^6, and 3×10^5 CFU/cm^2, respectively (Joseph et al., 2001). The same environmental materials can be found in a rendering facility; therefore, *Salmonella* biofilms can act as a continuous source for cross-contamination between contact surfaces and products.

A study conducted by Weigel et al. (1999) revealed some potential sources of the *Salmonella* contamination in animal feed on an Illinois swine farm. During the study, one hundred samples of feed were collected and only 2% were contaminated with *Salmonella*. In addition to the feed, samples were collected from the worker's boots, water, the floor, cats in the area, mice on the farm, and flies. The boots of the workers had the highest rate (17.2%, n=93) of *Salmonella* contamination, while the flies and the flooring had the lowest levels of contamination at 7.4% (n=95) and 7.9% (n=471), respectively. All of these mentioned factors could the potentially serve as a source for cross-contamination.

Salmonella has been identified as a common contaminant of rendered animal products. This has been reported in meat (81%) and feather meal (40%) by Hacking et al. (1977), Loken et al. (1968), and Beumer (1997) reported *Salmonella* contamination in sampled rendered products with incidence rates of 17% and 3.2-7.6%, respectively. The FDA conducted two separate studies in 1993 and 1994 to test for the presence of *S. enterica*, with *Salmonella* positive rates of 56% and 25%, respectively (McChesney et al., 1995; Crump et al., 2002). The Animal Protein Producers Industry (APPI) has collected weekly samples from the rendering processing plant and tested for the presence of *Salmonella*. The average incidence rate was reported as 25% (Franco, 2005). During one particular testing year, four serotypes commonly associated with food borne illness (Typhimurium, Enteritidis, Agona, and Infantis) were isolated with the prevalence rates of 0.5, 0.5, 3.0, and 3.5% of the samples, respectively. In addition, human *Salmonella* infection has been linked to the contaminated animal feed (Crump et al., 2002). Knox et al. (1963) established a connection to a *S.* Heidelberg outbreak from contaminated milk to meat and bone meal used in the feed supplied to the milk-producing cattle.

Salmonella: Survival Adaptations

Salmonella spp. are capable of adapting to numerous environmental stresses including extreme temperatures (high and low), a wide range of pH values, and low water activity (a_w) levels. Some strains of *Salmonella* have been able to grow in environments as high as 54°C and as low as 2°C (D'Aoust et al., 1975). Angelotti et al. (1961) detected *Salmonella* in custard and chicken a la king that had been heated to 45.6°C. Droffner and Yamamoto (1992) determined that a strain of *S.* Typhimurium was capable of mutating to survive at 54°C after prolonged exposure to thermal stress. The mechanism behind this resistance has not been determined, but the mutation has been determined to occur in the *ttl* gene or the *mth* gene which gives resistance

up to 48° and 54°C, respectively. In addition to these genes, other environmental factors play a role in the level of heat resistance. For example, heat resistance has been shown to increase as a result of a decrease in the a_w of the substance (D'Aoust, 1989). Goepfert demonstrated that when the substrate's a_w was reduced to a level of 0.90 by sucrose or glycerol, the *S.* Typhimurium had D-values at 57.2°C of 40-55 minutes and 1.8-8.3 minutes, respectively. Other factors that determine the level of heat resistance acquired include the nutrients available in the growth environment, the phase of growth the cells are in, and the moisture level of the environment it was isolated from (Goepfert et al., 1970; Kirby and Davies, 1990; Ng et al., 1969). *Salmonella* can also become heat resistance through continuous exposure to higher sub-lethal temperatures, a heat shock phenomenon. Exposing *Salmonella* cells to temperatures ≤50°C for 15-30 minutes results in the production of heat shock proteins that confer higher levels of heat resistance (Humphrey et al., 1993; Mackey and Derrick, 1990; Mackey and Derrick, 1986). The fate of *Salmonella* under freezing temperatures is dependent on the matrix it is suspended in, the kinetics of the freezing process, the physiological state of the bacterial cells before freezing, and the particular serotype involved (Corry, 1971).

In addition to growing or surviving under temperature extremes, *Salmonella* can adapt to a wide range of pH levels from 4.5 to 9.5 with an optimal range of 6.5 to 7.5. *S.* Typhimurium has been shown to acquire high levels of acid tolerance through repeated exposure to mildly acidic environments (ph 5.5-6.0). These exposed cells could then be transferred to a more acidic environments (pH ≤4.5) resulting in a complex acid tolerance reaction involving the production of acid shock proteins, new outer membrane proteins, reduced growth rate, and pH homeostasis which allows the cells to grow in environments with a pH of 3.0-4.0 (Foster and Hall, 1991; Hickey and Hirshfield, 1990). Three systems have been described that confer increased acid

tolerance to *Salmonella spp.* including: pH dependent and *rpoS* independent log phase acid tolerance response (ATR), the pH dependent and *rpoS* independent stationary phase ATR, and the pH independent and rpoS dependent stationary phase ATR.

Salmonella Infection and Disease

Salmonella infections have a wide array of symptoms depending on the serotype involved including enteric fever, enterocolitis, and potential systemic infections. Enteric fever results from infection with the typhoid or paratyphoid strains with symptoms including diarrhea, fever, headache, and prostration (D'Aoust, 1991). Those infections caused by non-typhoid strains typically results in enterocolitis which appears from 8 to 72 hours after infection. These infections are usually self-limiting resulting in diarrhea and abdominal pain that can last for up to 5 days (D'Aoust, 1989). In certain cases *Salmonella* can result in the following chronic conditions: aseptic reactive arthritis, Reiter's Syndrome, and ankylosing spondylitis.

McCullough and Eisele (1951) observed that a high level of *Salmonella* contamination (10^4-10^7 CFU/ml) was needed in egg nog to comprise an infectious dose; however, D'Aoust et al. (1985) and Kapperud (1990) have both demonstrated that 1 to 10 cells can be an infectious dose in food with a high solid fat content. A lower infectious dose is required in a food substrate with a high fat content due to the potential protection offered against the acidic environment of the stomach. Once inside the duodenum, the lipids compromising the fat would disperse and the *Salmonella* would be free to attach to the small intestine.

The first step of infection begins when *Salmonella* cells attach and then enter the epithelial cells (enterocytes) lining the intestinal tract and the M cells that overly the Peyer's patches. Attachment is brought on by the interaction between type 1 (mannose-sensitive) or type 3 (mannose resistant) fimbriae adhered to host receptors which are located on the microvilli or to

the glycocalyx surrounding the surface of the intestinal cells (D'Aoust, 1991, Polotsky et al., 1994). Upon contact with the epithelial cells, proteinaceous appendages form on the surface of the *Salmonella* cells (Galan and Ginnochio, 1994; Ginnochio et al., 1994). The production of these appendages is energy dependent and they are shed once membrane ruffles are formed on the attached epithelial cell (Ginnochio, 1994; Collazo et al., 1995). The attachment by the *Salmonella* cells initiates a series of chemical signals between the invading *Salmonella* cell and the host enterocyte or M cell (Garcia-del Portillo and Finlay, 1994; Ginnochio et al., 1994). The invasion (*inv)* locus stimulates a calcium influx which results in a rearrangement of the cytoskeleton within the host enterocytes and M cells (Finlay, 1994; Polotsky et al., 1994). The influx of calcium ions is responsible for actin polymerization (Ruschkowski et al., 1992). In addition to the calcium influx, the *Salmonella* cells also deliver the following invasion proteins to the host cells: SipA, SipB, SipC, SptP, SopE2, and SopB (Collazo and Galan, 1997; Collazo et al., 1995). Like calcium, these proteins stimulate the polymerization of actin into microfilaments in the vicinity of the invading *Salmonella* cell (Galan and Zhou, 2000; Ginnochio et al., 1992). Specifically, SipA and SipC act as catalyst for the polymerization of F-actin into microfilaments (Hayward and Koronakis, 1999.; Zhou et al., 1999; Zhou et al., 1999a). SopE2 activates the process of cytoskeleton rearrangement (Stender et al., 2000). The epithelial cell membranes undergo "ruffling" by which the membrane stretches to surround and engulf the attached *Salmonella* cell resulting in the bacterial cell entering the host epithelial cell via pinocytosis (Jones et al., 1993). Once the *Salmonella* cell has invaded the host cell, SptP results in the disruption of the host cell cytoskeleton returning into to its original state (Fu and Galan, 1998).

Once inside the cell, the *Salmonella* cell is initially confined inside a vacuole where it will initiate its replication (Garcia-del-Partillo and Finlay, 1994). The vacuole then is transported

through the cell, where it will eventually release the *Salmonella* cells into the lamina propria (Isberg and Nhieu, 1994; Polotsky et al., 1994). The fimbriae surrounding the *Salmonella's* surface allows the cells to attach to host plasminogen which could greatly enhance the invasiveness of that strain. Once attached, the plasminogen (zymogen) would likely be converted to its active proteolytic form where it would allow the bacterial cell to easily breach through host tissue barriers and penetrate into the deep tissues of the host (Sjobring et al., 1994).

Within the host cells, these *Salmonella* cells are capable of evading the host immune system by preventing phagosome acidification (Alpuche-Aranda et al., 1994; Alpuche-Aranda et al., 1992), avoiding lysosome fusion (Buchmeier and Heffron, 1991; Uchiya et al., 1999), or preventing the maturation of phagolysosomes (Rathman et al., 1997). Evidence has also demonstrated that *Salmonella* is capable of not only surviving inside phagolysomes but are capable of replication inside them as well (Oh et al., 1996; Rathman et al., 1996). For example, *Salmonella* produce several enzymes such as superoxide dismutase, peroxidase, and catalase that protect them from the toxic oxygen products formed during the metabolic burst of phagocytes. In total, there are 30 proteins produced in response to these toxic oxygen by-products (Popoff and Noral, 1992). *Salmonella* cells may also have an additional system that protects them from phagocytic cells referred to as the *phoP/phoQ* regulon (Miller, 1991; Popoff and Noral, 1992). It is believed that these genes are responsible for producing chemical signals which alter the core antigen component of the lipopolysaccharide (LPS) on the cell's outer membrane (Vaara et al., 1981) and provide resistance to antimicrobial peptides produced by neutrophils (Shafer et al., 1984). The *phoP/phoQ* gene set is also responsible for the production of a cell surface protease that degrades the defensins released by phagocytic cells (Guina et al., 2000).

Salmonella: Traditional Chemical Control Methods

Since *Salmonella* is a continuous problem for the food industry, methods of sanitation have been developed to reduced the risk of contamination in food products. Traditionally these methods of sanitation have employed the use of chemical disinfectants to clean surfaces the processed food may come in contact with. These chemicals have included chlorine based compounds, iodine containing compounds, and quaternary ammonium compounds (Cramer, 2006; Hui et al., 2003; Marriot,1994; Guthrie, 1992).

Chlorine based compounds are used as either a liquid chlorine, hypochlorites, chlorine dioxide, or inorganic and organic chloramines. The exact mechanism behind chlorine's activity is not clear but the following mechanism have been suggested: disruption of protein synthesis, oxidative decarboxylation of amino acids, reactions with the nucleic acids, disruption of metabolism by degrading enzymes, disruption of DNA, formation of toxic derivatives of cytosine, and creation of chromosomal aberrations (Marriot, 1994). These chlorine based compounds are corrosive and result in bleaching, so they must be used carefully around metals and material susceptible to bleaching. The biggest drawback to chlorine based compounds in the food industry is their affinity to bind to organic material. Once bound to this organic material the compounds are inactivated, so higher concentrations in the range of 100 to 150 ppm are required (Guthrie, 1992). The FSIS has required that animal slaughtering houses spray down their equipment with a solution containing at least 200 ppm chlorine (Swacina, 1988).

There are three forms of iodine primarily used in food processing sanitation i.e., iodophors, alcohol-iodine solutions, and aqueous iodine solutions. The bactericidal activity has not been studied in great detail but in general the iodine compounds release triciodide ions which mix with acid to produce hypoiodous acid and diatomic iodine, which are the active

antimicrobial forms (Marriott, 1994). Iodine containing compounds have drawbacks similar to those of chlorine. They are corrosive to metal and have a high affinity for organic material, which results in their inactivation. A positive aspect of these iodine-containing compounds is that they react with microorganisms rapidly and are effective against a wide range of organisms. These compounds are always mixed with detergents within an acid medium. The final solution should contain between 25 to 50 ppm active iodine (Guthrie, 1992).

The major form of quaternary ammonium compounds is cationic detergents which inhibit cell metabolic processes by inhibiting enzyme activity and cause the bacterial cells to leak resulting in lysis. It is also known that when these compounds are added to a surface they form a bacteriostatic film that is selective for particular microbes (Marriott, 1994). The quaternary ammonium compounds have the advantage that they are not corrosive, but unlike the previous two compounds, these quaternary ammonium compounds do not sufficiently work against gram negative bacteria. These compounds also have some additional limitations including: they tend to adhere to surfaces requiring excessive rinsing, they react with calcium and magnesium (cannot be used in hard water), and they are incompatible with most soaps and detergents (Guthrie, 1992).

Another class of sanitizer is acids. Acids used most frequently for food processing sanitation are the organic acids, such as acetic, peroxyacetic, lactic, propionic, and formic acids. These acids destroy the microbial cells by penetrating their cell envelope and acidify the cell's interior. The efficiency of these sanitizers are dose dependent and have great potential on surfaces where they may persist for long periods of time such as stainless steel (Marriott, 1994). The major disadvantage to the use of acid sanitizers is that they are highly corrosive (Guthrie, 1992).

Bacteriophages: Overview and Discovery

Bacteriophages have been used as a biological control method for various applications. Bacteriophages are viruses which use bacteria as a host for undergoing replication. The bacteriophages attach to specific bacterial cells and releases endolysins which degrade the bacterial cell wall. This allows the bacteriophage DNA to enter the cell where it hijacks the host's metabolic machinery to produce phage particles. The particles then self assemble and newly formed bacteriophages escape the host through cell lysis. These newly produced bacteriophages can then repeat this process with additional bacterial host cells (Doyle 2007).

Bacteriophages can be divided into five groups: tailed bacteriophages, polyhedral DNA bacteriophages, polyhedral RNA bacteriophages, filamentous bacteriophages, and pleomorphic bacteriophages (Ackermann, 2005). Of these the tailed bacteriophages account for the majority with ca. 4,950 different strains being identified (Ackermann, 2001). The tailed bacteriophages are all within the order *Caudoviridae* and are divided into three families based on their tail structure. These families include *Myoviridae* (contractile tail), *Siphoviridae* (long, non-contractile tail), and *Podoviridae* (short, non-contractile tail) (Ackermann, 2001).

The tailless bacteriophages only account for 4% of the identified bacterial viruses (Ackermann, 2001). The first type of tailless bacteriophage is the polyhedral bacteriophages, which contain an icosahedral head and have cubic symmetry (Ackermann, 1999). The polyhedral bacteriophages include *Microviridae* (ssDNA), *Corticoviridae* (dsDNA), *Tectiviridae* (dsDNA), *Leviviridae* (ssRNA), and *Cystoviridae* (dsRNA) (Ackermann, 2005). The second group of tailless bacteriophages is the filamentous bacteriophages, which include the families *Inoviridae* (ssDNA), *Lipothrixviridae* (dsDNA), and *Rudiviridae* (dsDNA) (Ackermann, 2005). The final type of tailess bacteriophage is the pleomorphic bacteriophages, which includes the families

Plasmoviridae (dsDNA) and *Fuselloviridae* (dsDNA) (Ackermann, 2005). Common characteristics of the different bacteriophages are summarized in Table 1.10.

Table 1.10 Characteristics of identified bacteriophage groups[1]

Shape	Family	Nucleic Acid	Adsorption Site	Infection by	Host-Virus Relationship	Example	Members
Tailed	*Myoviridae*	dsDNA	cell wall, capsule, pili, flagella	DNA	lytic or temperate	T4	1243
	Siphoviridae	dsDNA	cell wall, capsule, pili, flagella	DNA	lytic or temperate	Lambda	3011
	Podoviridae	dsDNA	cell wall, capsule, pili, flagella	DNA	lytic or temperate	T7	696
Polyhedral	*Microviridae*	ssDNA	cell wall	DNA	lytic	ØX174	40
	Corticoviridae	dsDNA	cell wall	?	lytic	PM2	3
	Tectiviridae	dsDNA	pili, cell wall	DNA	lytic	PRD1	18
	Leviviridae	SSRNA	pili	RNA	lytic	MS2	39
	Cystoviridae	dsRNA	pili, cell wall	capsid	lytic	Ø6	1
Filamentous	*Inoviridae*	ssDNA	pili	virion	temperate	fd	57
	Lipothrixviridae	dsDNA	pili	?	temperate	TTV1	6
	Rudiviridae	dsDNA	cell wall	?	carrier state	SIRV-1	2
Pleomorphic	*Plasmaviridae*	dsDNA	plasma membrane	DNA	temperate	L2	7
	Fusseloviridae	dsDNA	?	?	temperate	SSV1	7

1(Ackermann, 2005)

The idea of using phage as a form of treatment has gained significant interest as an alternative to antibiotics. The majority of antibiotics (50-70%) used in the United States are given to farm animals as a prophylactic, to treat livestock, or as a growth promoter (Gustafson, 1991). The overuse of these antibiotics may have contributed to the decline in efficiency of the antibiotics in humans, but banning their use completely may have a negative effect on the health of the animals they have been administered to (Kutter and Sulakvelidze, 2005). An alternative method of treatment, such as bacteriophage therapy, is necessary. The use of phage offers multiple advantages: the host specificity is not likely to select for phage resistant bacterial strains, resistance against phage does not affect the effectiveness of antibiotics given to humans, and phage mixtures can be easily modified and rapidly developed against resistant bacteria (Kutter and Sulakvelidze, 2005).

The use of bacteriophage as a therapeutic agent was first realized by Felix d'Herelle in 1919. D'Herelle was conducting research to determine if phage could be used to prevent the natural infection of chickens by *Bacillus gallinarum*. The phage appeared to prevent development of avian typhosis, the disease caused by *B. gallinarum*. The treatment was then performed in large scale in rural areas of France where the disease had developed into an epidemic. The flocks of chickens that received the phage treatment "had fewer deaths, the duration of the epidemic was shorter, and second rounds of infection were prevented" (d'Herelle, 1921; Kutter and Sulakvelidze, 2005). D'Herelle also demonstrated that the administration of phage to water buffalo in

Indochina could prevent infection with *Pasteurella multocida*, the causative agent of bovine hemorrhagic septicemia (d'Herelle, 1926). The use of bacteriophages as a form of therapy has been investigated well before the introduction of antibiotics. In the 1930s, such therapy was being marketed by American pharmaceutical companies. During World War II, the Germans and Soviets were using phage therapy to treat dysentery; however, the advent of antibiotics in the 1940s caused a decline in the need for research with phage therapy. Today with the prevalence of antibiotic resistant bacteria on the rise, bacteriophage research is on the rise as well especially in terms of biopreservation of food products. Bacteriophages naturally found in the food microbiota are safely consumed as part of the human diet. It is possible then that bacteriophages can be used to reduce or eliminate contamination by foodborne pathogens such as *Salmonella* (Doyle 2007).

Life Cycle of Bacteriophages

Bacteriophages have two alternative life cycles, lysogenic or lytic. In both cycles, the virus will adhere to specific receptors on the bacterial cell's surface, where it will penetrate the surface and inject its nucleic acid into the host cell. Once the genetic material is inside the cell, the bacteriophage will enter one of two life cycles. At this point the bacteriophage may enter the lysogenic phase by inserting the nuleic acid into the chromosome of the bacterial host. This state is referred to as a prophage. The prophage is replicated along with host cell replication so that all progeny will contain the bacteriophages genetic material as well. The bacteriophage will remain in this prophage form until it is induced to leave the host chromosome, replicate, and lyse the cell. The

second route for the bacteriophage is the lytic cycle, which is the focus of this study and discussed in more detail in the following paragraphs (Cowan and Talaro, 2009).

Bacteriophage infection begins with the attachment of the bacteriophage virus to the bacteria's surface receptors. Attachment is initiated when the tail fibers or spikes of the virus recognize and bind to specific surface receptors of its bacterial host. In gram-negative bacteria numerous components can serve as a surface receptor including any of the outer membrane proteins, the lipopolysaccharides, and the oligosaccharides. While some bacteriophages only require a single attachment type of receptor, others such as T4-like phages go through a two-part attachment process involving two types of receptors. In the case of the T4-like bacteriophages, at least three of the six tail fibers have to bind to their surface receptors which trigger the rearrangement of the baseplate proteins in the phage allowing the spike to irreversibly bind to the host's surface (Guttman et al., 2005).

Following attachment, the bacteriophage penetrates the surface of the host cell and injects its genetic material into the cell. The "injection" process actually involves a series of mechanisms for the transfer of DNA that is specific for each bacteriophage. The general process initiates when the tail pins release enzyme that allow them to break through the cell wall and inner membrane. Once this opening has been made the bacteriophage's genetic material is released into the cell. The bacteriophage also has mechanisms that prevent the genome from leaving the capsid prematurely. The process of how the DNA enters into the host cell is poorly understood and is dependent on the bacteriophage involved. For example, DNA enters the host cell from T7 phages as a result of its transcription. Once inside the cell, the bacteriophage genome is susceptible to

degradation by host cell enzymes, so bacteriophages have evolved mechanisms to protect their genetic material. One way in which they do this is by circularizing their DNA through the use of terminal redundancies ("sticky ends"). Some have incorporated an irregular nucleotide that is not commonly found in DNA or RNA such as hydroxymethyldeoxyuridine or hydroxymethyldeoxycytidine which are found in SP01 and T4 bacteriophages, respectively. As another mechanism, some phages such as the Streptococci phage SD-1 and the coliphage N4 have evolved so they no longer contain sites that are commonly recognized by enzymes of their natural host bacterial species (Guttman et al., 2005).

Inside the host cells, the bacteriophage genome is recognized by the host RNA polymerase leading to the initial transcription and translation of "early genes". The products of these genes are responsible for blocking host proteases and restriction enzymes, terminate host cell metabolism, and denature host proteins. After this is complete, a second set of "middle genes" is transcribed and translated to produce the products needed for replication of the bacteriophage genome. Following replication, a final set of "late genes" is transcribed and translated to produce the protein components of the bacteriophage particles. In all of these processes, the bacteriophages either produce sigma factors or DNA binding proteins to reprogram the host cell RNA polymerase or they contain a gene set that is transcribed and translated early that codes for its own RNA polymerase. Additional adaptations include the degradation of the host DNA and preventing protein synthesis (Guttman et al., 2005).

Following replication and the production of components of the bacteriophage particles, the bacteriophages have to be assembled and the genetic material needs to be packaged within the capsid head. The packaging of the capsid protein involves interactions between scaffolding proteins and structural proteins. At one vertex of the icosahedral head there is a portal complex which is the origin site for head assembly, docking site for packaging enzymes, and the site at which the genome enters the capsid. At some point during the packaging process, the head expands in order to accommodate the bacteriophage DNA or RNA (Guttman et al., 2005).

Once the bacteriophages have completely assembled, they must exit the cell through lysis. The process of lysis is dependent on the bacteriophage type and its timing is crucial (Guttman et al., 2005). If the cell is lysed too soon, there is not enough bacteriophages to continue the lytic cycle, but prolonging lysis too long results in the loss of opportunities for infection (Abedon, 1990). Tailed phages use a two enzyme system for lysis. They produce pores in the inner cytoplasmic membrane by an enzyme. The second enzyme, lysin, passes through the pores formed by holin and begins breaking down the bonds between the layers of peptidoglycan. The tail-less phages produce individual enzymes that inhibit the host cell enzymes responsible for peptidoglycan formation (Guttman et al., 2005). The lytic process is illustrated in Figure 1.2

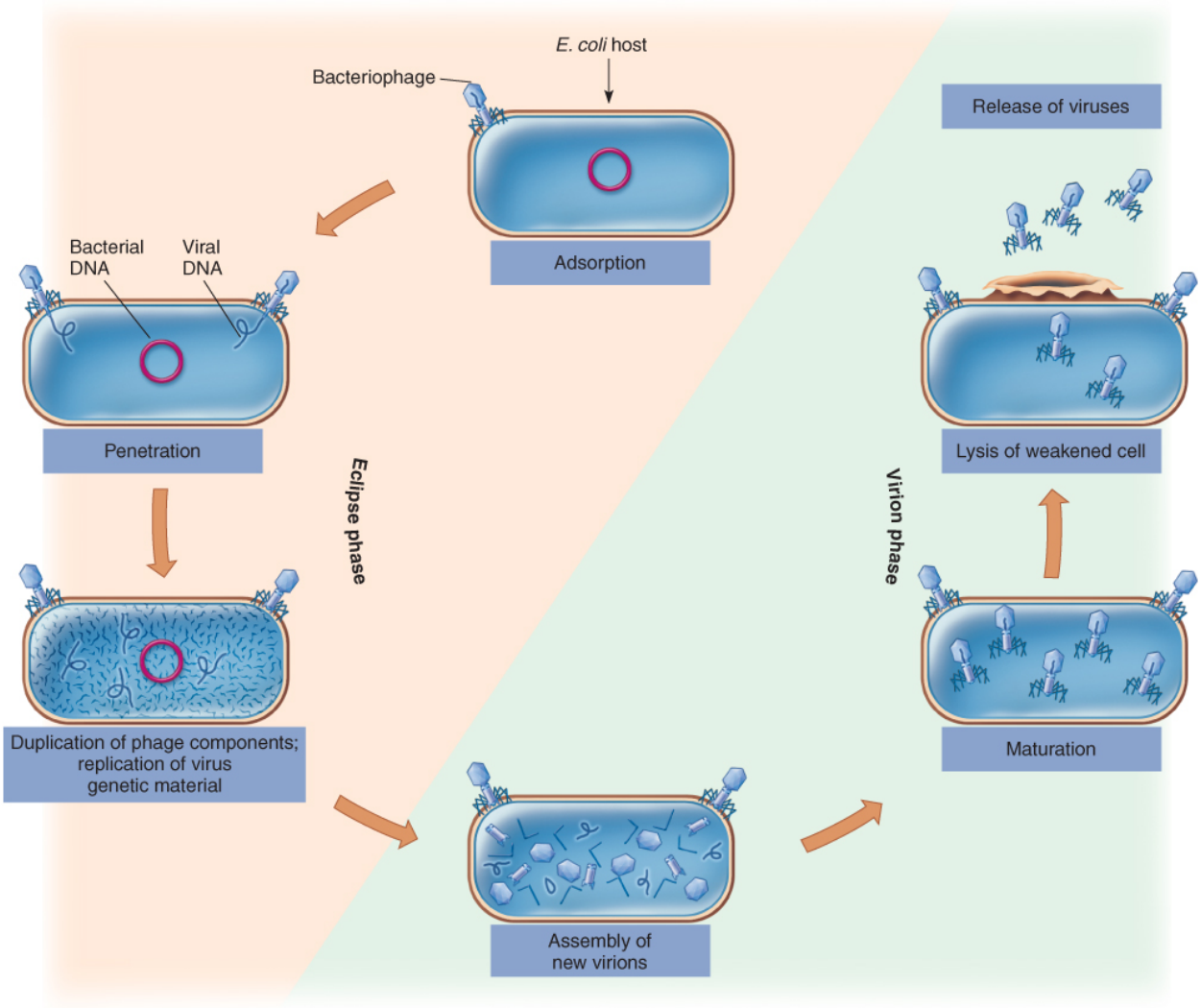

Figure 1.2 Bacteriophage lytic cycle (Cowan and Talero, 2009)

Bacteriophage Treatment of Raw Meats and Surfaces

While there is a lack of studies investigating phage usage as a treatment method in animal by-products, several researchers have demonstrated the possibility of creating bacteriophage mixtures capable of reducing *Salmonella* levels significantly in the raw animal meats. One group of researchers found that phage treatment can significantly reduce *S.* Enteritidis (SE) levels in poultry products (Higgens et al., 2005). The study

found that the phage PHL4 (10^8 and 10^{10} PFU/ml) was capable of reducing SE on the carcass by 85-93% and in the rinse water by 50-100%. In addition, a 72 phage cocktail (10^7-10^8 PFU/ml) used to treat SE on commercially processed turkey carcasses resulted in a 50-60% reduction in SE. Another study demonstrated that SE (10^6 CFU/ml) contamination on chicken skin was significantly reduced by washing the skin with 10^9 PFU/ml of three wild-type bacteriophage cocktail (Fiorentin et al., 2005). During storage at 5°C, a significant difference between the means of *Salmonella* populations on the control and experimental skin was observed on days 3, 6, and 9 with *Salmonella* reduction at 2.27, 4.56, and 4.49 x 10^8 (CFU / cut), respectively.

Studies have shown bacteriophages to be effective against *Salmonella* serovars other than Enteritidis as well. Atterbury et al. (2007) recovered 232 phages from poultry farms, abattoirs, and wastewater from 2004-2005. Among those collected phages, the three with the broadest range against Enteritidis, Hadar, and Typhimurium were selected for further study. The phage cocktail resulted in $\geq$ 4.2 and $\geq$2.19 $\log_{10}$ CFU reduction in Enteritidis and Typhimurium cecal colonization, respectively (Atterbury et al., 2007).

There are also studies demonstrating the ability of bacteriophage to reduce the population of bacterial cells attached to an inert surface. Roy et al. (1993) studied the effectiveness of phage treatment against *L. monocytogenes*. In their study, a three phage mixture (H387, H387-A, and 2671) was used to treat stainless steel templates that had been contaminated with the pathogen. Phage treatment resulted in a 3 log decrease in *L. monocytogenes* populations. The elimination of *L. monocytogenes* from stainless steel was also investigated by Hibma et al. (1997).

Bacteriophage Treatment of Biofilms

Often bacteria are not simply attached to environmental surfaces but are rather components of a large microbial network known as a biofilm. A biofilm can be composed of one or various species of bacteria and their excreted products. The concern for phage treatment is to ensure that the phage can actually come in direct contact with the bacterial cells within the biofilm matrix. It has been shown that many biofilms actually contain water filled pathways that can transport the phage into the biofilm's interior and provide access to the bacteria cells. In addition, a large number of phages are capable of producing polysaccharases or polysaccharide lyases. These enzymes are host specific so they are only effective against a range of related polysaccharide structures. Phages capable of degrading the exopolysaccharide (EPS) of various gram-negative bacteria, including species that are capable of producing a biofilm, have been isolated (Sutherland, 2004).

Studies on the effect of phage on biofilm produced by *Enterobacter agglomerans* revealed that the biofilm can be degraded if two conditions were met (Hughes, 1998a; Hughes, 1998b). First the bacterium being investigated has to be susceptible to the phage being used. Second the polysaccharide lyases produced by the phage has to be able to degrade the biofilm's EPS. Even when only one of these conditions were met, the phage still cause a significant amount of biofilm degradation.

The effects of phage treatment on single species biofilms have been well documented. Doolittle et al. (1996a) discovered that the matrix produced by an *E. coli* biofilm was ineffective in preventing phage T4 from infecting the bacterial cells. To

confirm their findings, the T4 phage was labeled with fluorescent and chromagenic probes and introduced to the *E. coli* biofilm. Microscopic observations revealed that the T4 was infecting the *E. coli* cells and were not inhibited by any of the extracellular components within the biofilm (Doolittle et al., 1996b). Karunasagar et al. (2007) demonstrated that phage can be used to reduce *Vibrio harveyi* levels in biofilm formed on high density polyethylene. When using a phage concentration of 10^5 PFU/ml, *V. harveyi* counts were reduced nearly 1 log CFU/cm^2 after 18 h. A higher phage concentration (10^8 PFU/ml) reduced the *V. harveyi* counts by 1 log every 6 h from 2.19 x 10^6 to 9.40 x 10^3 CFU/cm^2 in 18 h. Another study determined the effectiveness of phage KH1 against *E. coli* O157:H7 biofilm (Sharma et al., 2005). The phage was able to significantly reduce ($P<0.05$) bacterial counts in biofilms produced from strain FRIK 816-3 from an initial concentration of 4.0 log CFU per coupon to 2.8 log CFU per coupon and 2.7 log CFU per coupon after 24 and 48 h of phage treatment, respectively.

Research on the use of phage for biofilms composed of multiple bacterial species has not been thoroughly investigated, and some reported less promising results (Sutherland 2004). Storey and Ashbolt (2001) attempted to treat biofilm composed of *Bacteroides fragilis* and *E. coli* in an urban water distribution system with phages. The phages were initially taken in by the biofilm that had formed on stainless steel and polythene surfaces; however, following the initial treatment, phage numbers began to decrease rapidly, suggesting that the phages were not infecting the host cells. Their study has shown that biofilms composed of multiple bacterial species may contain additional barriers such as bacterial cell co-aggregation (Rickard et al., 2003) that phage treatment

must overcome to be successful. Therefore, additional research into developing phage treatments against biofilms composed of more than one species of bacteria is needed.

Examples of *Salmonella* Reduction via Bacteriophage Treatment

Several studies have demonstrated that phage treatment can be very effective against *Salmonella* contamination. For example, a study conducted by Modi et al. (2001) investigated whether the addition of phage SJ2 to the starter culture used in cheddar cheese production would reduce the survival of *Salmonella*. A strain of *Salmonella* Enteritidis that had been associated with an outbreak due to contaminated cheese was inoculated into the milk post pasteurization at 10^4 CFU/ml. The results showed that the experimental cheese made with pasteurized milk was negative for *Salmonella* contamination after 88 days. The experimental cheese produced with raw milk treated with bacteriophage had very low *Salmonella* contamination levels of 50 CFU/g. In contrast, all cheeses produced without bacteriophage had *Salmonella* counts of 10^3 CFU/g after 99 days. Whichard et al. (2003) demonstrated that both the wild-type and a large plaque variant of the Felix 01 phage can be used to control *S.* Typhimurium contamination on chicken frankfurters. The results indicated both phage strains were capable of reducing *S.* Typhimurium levels by 10^2 CFU/g. A study by Leverentz et al. (2001) demonstrated the potential use of phages for reducing *Salmonella* contamination on fresh produce. Their results demonstrated that the phage was capable of reducing *Salmonella* levels on fresh cut honey-dew melon by 3.5 logs at 5°C and 10°C, and a 2.5 log reduction at 20°C. There was no significant reduction of *Salmonella* on apple slices at any temperature. Titers of the phage were stable on the honey-dew melon but were non-

detectable on the apples after 48h, this is possibly due to the more acidic pH (4.2) of the apples as opposed to that of the melon (5.8). Pao et al. (2004) studied the effect of two different phages simultaneously against three serotypes of *Salmonella*. One phage was effective against *S.* Typhimurium and *S.* Enteritidis. The other phage was effective against *S.* Montevideo. Phage treatment was shown to significantly suppress growth of *S.* Typhimurium and *S.* Enteritidis in both broccoli and mustard seeds for 24 h. A combination of the two bacteriophages suppressed the growth of the three serotypes by 0.57 ± 0.04 log ($P<0.001$) in the broccoli seeds. One desirable aspect of phage treatment is the host specificity of bacteriophages. In terms of *Salmonella* treatment, this host specificity is a hurdle for reducing contamination levels. There is not a single phage capable of lysing all known serovars of *Salmonella*. In fact, a particular phage may not even be capable of lysing all members of a particular serovar. To overcome this shortcoming, a "cocktail" of phages must be designed capable of lysing all *Salmonella* strains of interest (Joerger 2003). The production of an effective phage cocktail for *Salmonella* has been reported previously. For example, Chighladze et al.(2001) reported to have developed a phage cocktail capable of lysing 232 of 245 *Salmonella* isolates composed of 21 serovars with 78 pulse field gel electrophoresis (PFGE) pattern types (Joerger 2003).

Bacteriophage Therapy in Animals

As previously mentioned, the use of bacteriophages as a therapeutic agent dates back 1919 with the work of Felix d'Herelle (Sulakvelidze and Barrow, 2005). Salmonellosis in mice served as one of the earliest models for bacteriophage therapy. For

example, Topley et al. (1925) tested the effectiveness of bacteriophage therapy in mice infected with *S.* Typhimurium. The results indicated that the therapy did not reduce the mortality rate or shedding of the bacteria in the feces. The ineffectiveness during this study was contributed to the lack of optimization of the bacteriophage. The bacteriophage used was found not to completely lyse the bacterial strain (Topley and Wilson, 1925). Fisk's study (1938) concluded that *Salmonella* infection could be prevented through the use of bacteriophage therapy.

The interest in bacteriophage as a therapeutic agent was renewed by the work of Smith and Huggins (1982). Their initial studies investigated the effectiveness of bacteriophages specific to the K1 capsule antigen found on *E. coli* O18:K1:H7 ColV. Their results indicated that a single injection of the bacteriophage (3×10^8) was more effective than several antibiotics in protecting mice against infection by that *E. coli* strain. In a subsequent study, Smith and Huggins (1983) tested the ability of a bacteriophage to prevent infection in calves, piglets, and lamb by *E. coli* O9:K30.99. The mortality rates for calves, piglets, and lambs infected with *E. coli* and left untreated were 100, 57, and 25%, respectively; whereas, the mortality rate for those animals treated with the bacteriophage was 17, 0, and 0%, respectively.

The success of the *in vivo* studies by Smith and Huggins renewed the interest in bacteriophage therapy. More recently, Huff et al. (2002) conducted three experiments to determine the effect of bacteriophages on respiratory infections by *E. coli* in broiler chickens. During all three trials, *E.coli* was administered by air-sac inoculation while the bacteriophage was administered by either air-sac inoculation or in the drinking water.

The results showed that bacteriophage treatment by air-sac inoculation reduced the mortality rate from 80% to 0% or 30% depending on the concentration of bacteriophage used. Treatment with the bacteriophage in the drinking water demonstrated the bacteriophage was only effective when given prior to infection. When given prior to infection, the bacteriophage was able to reduce the mortality rate from 85% to 0% or 35% depending on the concentration of the bacteriophage. Biswas et al. (2002) demonstrated that other bacteria, such as VRE, can be treated with bacteriophage therapy as well. The bacteriophage was shown to prevent bacteremia caused by VRE infections in mice.

Recent studies have also tested the effectiveness of bacteriophage as a therapeutic agent against *Salmonella* as well. Berchieri et al. (1991) were able to isolate several phages with activity against *S.* Typhimurium. The isolated phages were then orally administered to chickens infected with *S.* Typhimurium. The addition of the bacteriophages was shown to significantly reduce the mortality rate. Phage 2.2 was one of the most effective isolates during their study. It decreased the viable numbers of the pathogen within the host's alimentary tract. The *Salmonella* population was decreased by 1 log in the crop, intestine, and caeca of the chickens within 12 h of phage administration. *Salmonella* counts in the liver were reduced by 0.9 log within 24 h of phage administration. The results also demonstrated that administering the bacteriophage close to the time of infection was significantly more efficient than delaying bacteriophage administration. This was believed to be due to the nature of *Salmonella* which is an intracellular pathogen. In theory, it is more effective to expose the *Salmonella* to the bacteriophage before it is able to enter the epithelial cells in the intestinal tract. This study

demonstrated that oral administration of bacteriophage to animals can reduce *Salmonella* contamination levels.

Advantages of Bacteriophage Treatment/Therapy

The use of antibiotics as feed additives has become a major concern for national health organizations and the livestock industry due to the increase in antibiotic resistant pathogens in human health that have been linked to those antibiotics used in livestock feed (Smith et al., 2002). This has led to the banning of many traditional antibiotics as feed additives. For example, the European Union has banned the use of virginiamycin, bacitracin zinc, spiromycin, and tylosin phosphate (Ferber, 2003). While the bans may have decreased the amount of antibiotic use, it also poses health concerns to the livestock which will lead to safety concerns in food products. An alternative treatment that is both effective and safe to the animal host and environment is needed to replace the antibiotics in the feed.

Bacteriophages may be the most environmentally safe alternatives available considering they are naturally found in the environment and animal hosts. In addition, they have several advantages over antibiotics. Typically the antibiotics used as feed additives are broad spectrum so as to target as many bacterial species as possible. The issue is when resistance arises it can occur in several different bacterial species. Bacteriophages have a high specificity for their host, typically only infecting one bacterial species or even a few serotypes within a species. An advantage with the use of bacteriophages is that if there is bacteriophages resistance it would only arise in the bacterial species that is their host and would have little to no effect on other species in the

environment. The resistance mechanism for bacteriophages is not related to the mechanisms developed in antibiotic resistance, so resistance to a bacteriophage will not influence the efficiency of antibiotics that are used for pathogen treatment. When resistance does occur, bacteriophages can be rapidly modified through natural selection to produce a new treatment; whereas, antibiotics may require several years of research and development to produce (Pirisi, 2000). In application, it has been demonstrated that bacteriophages may be transferred between animal hosts. This transfer provides a large advantage as a prophylactic due to the fact one animal could be administered the bacteriophage and it would transmit throughout the population on its own (Smith et al., 1987). A final advantage is that they are self-replicating, so a single dose would last throughout the course of infection. This advantage would also mean a relatively low initial dose could be used (Barrow and Soothill, 1997).

Summary

It is well established that finished rendered by-products can be contaminated with pathogenic bacteria including *Salmonella spp.* and *Enterococcus spp.* The reason behind this contamination is debatable and is one of the focuses of our study. Although the material may be contaminated, the rendering process is still the best alternative available for handling animal by-products, and it provides a valuable source of nutrients needed for the finished animal feed. Although the benefits of the feed may outweigh the biological risks, there is still concern that the feed serves as a continuous vehicle for introducing pathogenic bacteria into the food chain. In this particular study, the focus is on enterococci and *Salmonella*. Enterococci typically only cause mild treatable disease, so

the concern is with VRE which has the capability of transferring the vancomycin resistance genes to more pathogenic bacteria via conjugation.

Salmonella is one of the leading causes of human food-borne illness; therefore, its presence in finished meals is of major concern. The heat used during the rendering process is sufficient to kill pathogenic bacteria, which leads to the theory that the contamination was resulting from cross-contamination within the rendering facility. There are several potential sources for cross contamination including the incoming raw material and the environmental surfaces found within the facility.

Due the potential for cross-contamination, it is important to develop a method for reducing bacterial levels that is biologically safe and cost efficient. Bacteriophages could potentially serve this need to the fact that they are self-producing and only affect their determined bacterial host. Numerous studies have demonstrated the ability of bacteriophages as a treatment method for both raw meat and hard surfaces such as steel. Bacteriophages have also been show to be an effective means of preventing or reducing infection within an animal host.

The objectives of this study were as follows:

- Determining contamination levels of *Salmonella* spp. and *Enterococcus* spp. within the finished rendered products provided by U.S. rendering facilities.
- Characterizing and determining heat resistance of *Salmonella* and *Enterococcus* isolated from the animal by-products.
- Screening enterococci isolates for VRE and determining the level of resistance.

- Developing a bacteriophage cocktail capable of lysing *Salmonella* that had been recovered from finished by-products
- Using the produced bacteriophage cocktail to reduce *Salmonella* levels on environemental surfaces (steel, rubber, HDPE plastic, and concrete) in the form of attached cells and within a biofilm.
- Using the bacteriophage cocktail to reduce *Salmonella* levels in the raw material used to produce the rendered by-products.
- Conducting an animal study to use the bacteriophage cocktail as a feed additive for therapeutic purposes against *Salmonella* infection in mice.

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CHAPTER 2

ANALYSIS OF *SALMONELLA* AND ENTEROCOCCI ISOLATED FROM RENDERED ANIMAL PRODUCTS

Abstract

The objectives of this study were to determine the current status of bacterial contamination in rendered animal products and analyze *Salmonella* and enterococci isolates from the samples. One hundred and fifty samples were provided by various rendering companies across the U.S., including the following meal types: feather, meat, meat and bone, meat and bone from poultry, poultry, and blood meals. The average pH of the meals ranged from 6.16 to 7.36, and the moisture content ranged from 1.9 to 11.5%. The total bacterial counts were in the range of 1.7 to 6.68 log CFU/g with the highest in blood meal and the lowest in meat meal. *Enterococcus* species were detected in 81.3% of the samples and accounted for up to 54% of the total bacterial counts in some samples. Both blood meal and feather meal were more contaminated ($P < 0.05$) with enterococci than other meal types, although all blood meals were from a single company. *Salmonella* was detected in 8.7% of the samples. *E. coli* was not detected in any of the samples, but coliforms were detected in four samples. Among enterococci isolates, three were vancomycin resistant. Thirteen serotypes of *Salmonella* displayed 16 pulse field gel electrophoresis (PFGE) patterns. PFGE analysis has indicated that *Salmonella* contamination was not persistent in the plant environment over time. D-values for the *Salmonella* isolates at 55°C, 60°C, and 65°C were in the ranges of 9.27-9.99, 2.07-2.28, and 0.35-0.40 min, respectively. These results suggest that the presence of *Salmonella* in

the finished products may be due to post-processing contamination. This study has also revealed that the rendering industry has microbiologically improved their products as compared to earlier studies.

Introduction

Each year, the U.S. meat industry generates over 2.3×10^{13} kg of animal parts not typically included in the food supply such as fat and bone trimmings, meat scraps, blood, offal, and feathers (Franco and Swanson 1996). Annually, the U.S. produces ca. 11.2 billion pounds of protein and 10.9 billion pounds of fats. Approximately 85% of these products, are then used in the production of animal feed ingredients (Meeker and Hamilton, 2006). A large portion of these U.S. products are then exported. In 2005, the amount of exported finished products worldwide was ca. 1.7 million metric tons with 0.8 million metric tons being exported by the U.S. (Swisher, 2006). These by-products are often contaminated with high numbers of microorganisms and will spoil rapidly becoming a public health hazard if left untreated. Meat, meat and bone, feather, poultry and blood meals are produced by the rendering industry from these by-products of meat processing plants. Therefore, the rendering industry has played a significant role in recycling inedible parts of food animals into a variety of value-added products (Franco and Swanson 1996). Most importantly, the rendering industry contributes to maintaining a healthy environment by using processing conditions capable of eliminating pathogenic microorganisms including bacteria and viruses.

While adding rendered animal meals to feed provides many needed nutrients and minerals essential for animal growth, animal feed is a source of foodborne pathogens contributing to human foodborne illnesses (Crump et al. 2002). Although the rendering process involves high levels of heat treatment, there is still potential for the finished meal to become contaminated with bacteria. When these contaminated products are used as ingredients of animal feed, inoculation of other animals in the food chain can occur and lead to potential human exposure. A previous study on rendered products revealed poultry meal containing bacterial populations ranging from 1.6 to 4.02 log CFU/g (Hofacre et al. 2001). About 85% of the poultry and meat and bone meal samples contained bacteria resistant to amoxicillin, ampicillin, clavulanic acid and cephalothin. Some of the most commonly isolated bacteria were *Acinetobacter colcoaceticus, Citrobacter freundii,* and *Enterobacter cloacae*. *Salmonella* was also isolated from 14% of the meat and bone samples (Hofacre et al. 2001).

Enterococci have often been used as probiotics in food production, but also account for 12% of the nosocomial infections in the U.S. causing bacteremia, endocartitis, and infections, with *Enterococcus faecalis* and *Enterococcus faecium* causing the majority of these infections (Franz et al. 2003). Enterococci are resistant to various stresses including mild heat treatment and are intrinsically resistant to a wide variety of antibiotics. More specifically, resistance to vancomycin is of major concern since vancomycin is considered to be the last resort for enterococci treatment (Manero et al. 2006, Wegener 2003). Therefore, it is important to identify potential reservoirs for enterococci in an effort to control the possible contamination with this microorganism.

The purpose of this study is two-fold. First it is necessary to determine if the rendered meals may serve as a vehicle for bacteria, specifically *Enterococcus* spp. and *Salmonella* spp. Although early studies have demonstrated that rendered products are frequently contaminated with bacteria including *Enterococcus* spp. and *Salmonella* spp., the technological improvements of the rendering process have been made, and an updated study is needed to assess the microbiological quality of these products by taking rendered products directly from the production line to reduce the potential for post-processing contamination. The study then determined whether the contamination is likely to be a result of insufficient sterilization methods or through post-processing contamination.

Materials and Methods

Analysis of moisture content, pH, and water activity. To determine the moisture content, approximately 1g of each sample was weighed and dried in a mechanical convection oven (Blue M Electric Co., Blue Island, IL.) at 105°C for 24 h. For pH testing, one gram of each sample was mixed with 50 ml of distilled water and analyzed using a pH meter (Orion perpHect Log R meter model 310, Boston, MA.). The water activity (a_w) values were determined using a Rotronic-Hygroskop DT (Rotronic Instrument Corp., Huntington, NY.).

Bacterial enumeration. One hundred and fifty different rendered samples were provided by twelve rendering companies throughout the U.S from August, 2004 to February, 2005. The products were separated into six meal types: feather meal (n=30), meat meal (n=14), meat and bone meal (MBM) (n=37), meat and bone meal from poultry

(MBM-P) (n=6), poultry meal (n=51), and blood meal (n=12). Samples were stored at 4°C prior to analysis.

Samples in duplicate were serially diluted using 0.1% sterile peptone water. Aliquots of each dilution were spread-plated onto Tryptic Soy Agar (TSA, Becton-Dickinson and company, Sparks, MD.) for total bacterial counts (TBC) and Bile Esculin Agar (BEA, Becton-Dickinson and company) for enterococci counts, separately. Plates were incubated at 37°C for 24 h. Bacterial counts were reported as CFU/g, and the detection limit for both TBC and enterococci was 100 CFU/g.

Detection of *E. coli*/coliforms and *Salmonella*. Ten grams of each sample in duplicate were mixed with 90 ml of universal pre-enrichment broth (UPB, Becton-Dickinson and company) and incubated at 37°C for 24 h. For *E. coli*/coliforms testing, 1 ml of each enriched sample was placed on a Petrifilm™ *E.coli*/coliforms count plate (3M Microbiology Products, St. Paul, MN.) and incubated at 37°C for 48 h. For *Salmonella* detection, the enriched sample was inoculated into Tetrathionate Broth (TTB, Becton-Dickinson and company) and incubated at 37°C for 24 h, followed by plating onto xylose lysine tergitol-4 (XLT-4, Becton-Dickinson and company). Presumptive *Salmonella* isolates were then confirmed with a *Salmonella* identification latex test (Oxoid®, Basingstoke, Hants, UK). The above procedure deviated some from FDA recommendations due to the quantity of samples received. Standard procedures require a 25 g sample, but some samples were not large enough to do so.

Bacterial isolation. Purified isolates from both BEA (*Enterococcus*, n=200) and XLT-4 (*Salmonella*, n=30) plates were streaked onto TSA, preserved in TSB containing

20% glycerol, and stored at -80°C. These isolates were used for further testing including antibiotic and heat resistance determination.

***Enterococci* spp. confirmation.** The prospective enterococci isolates were gram-stained and then tested for growth at 45°C in TSB, growth in TSB containing 6.5% NaCl, catalase activity, and pyrrolidonyl arylamidase activity using PYR disks (Remel, Lenexa, KS.). All isolates that tested positive as an *Enterococcus* spp. were screened for vancomycin resistance by plating onto BEA supplemented with 6 µg/ml of vancomycin (Sigma-Aldrich, St. Louis, MO.) and incubating at 37°C for up to 48 h.

Determination of minimum inhibitory concentration (MIC) of vancomycin. The agar dilution assay was used as described by CLSI protocol (CLSI, 1997) using Mueller Hinton agar (MHA, Becton-Dickinson and company). Plates were prepared using the following concentrations of vancomycin: 4, 8, 16, 32, 64, and 128µg/ml. Enterococci isolates were inoculated onto the MHA plates using a replica plater device (Sigma). Plates were incubated at 37°C for up to 48 h.

PCR identification of genus and species for potential vancomycin resistance enterococci (VRE) isolates: The DNA of potential VRE isolates (n=16) were extracted by boiling method and used for genus confirmation and identifaction of the species *E. faecium* and *E. faecalis*. The primers, reaction mixtures, and reaction protocol were performed according to Shank et al. (2008), Sedgley et al. (2005), and Jackson et al. (2005), respectively. The PCR reaction was conducted in a Bio-Rad®iCycler iQ™ (Bio-Rad Laboratories, Hercules, CA), and the resulting products underwent electrophoresis in a 1.5% agarose gel at 70 v, and stained in ethidium bromide. Images of the gels were

captured using a GelDoc 2000 (Bio-Rad Laboratories). *E. faecalis* ATCC 29212, *E. faecium* ATCC 51229, and *E. coli* G5244 were used as controls.

Detection of *vanA* and *vanB* genes through PCR analysis. The DNA of potential VRE isolates (n=16) were extracted by boiling method and used to detect the presence of the *vanA* or *vanB* genes. The primers, reaction mixtures, and reaction protocol were performed according to Dutka-Malen et al. (1995). The PCR reaction was conducted in a Bio-Rad®iCycler iQ™ (Bio-Rad Laboratories, Hercules, CA), and the resulting products underwent electrophoresis in a 1.5% agarose gel at 70 v, and stained in ethidium bromide. Images of the gels were captured using a GelDoc 2000 (Bio-Rad Laboratories). *E. faecium* ATCC 51559 (vanA), *E. faecium* ATCC 51229 (vanB) and *E. coli* G5244 were used as controls.

Thermal resistance screening. Bacterial isolates (*Salmonella*, enterococci, and VRE) were screened for thermal resistance. All cultures were grown on TSA at 37°C for 24 h, and each culture was resuspended in saline and inoculated into TSB at a final concentration of 5×10^6 CFU/ml. The inoculated TSB tubes were incubated at 60° and 70°C in a Haake V26 circulating water bath (Thermo Haake GmbH, Karlsruhe, Germany) for *Salmonella* and enterococci, respectively. Three tubes were removed simultaneously at pre-determined time intervals and placed immediately into an ice bath. After the tubes had cooled, each tube was incubated at 37°C for 24 h and observed for the presence of growth.

D-value and z-value determination. The six most heat resistant *Salmonella* isolates were inoculated into TSB and incubated at 37°C for 24 h. After incubation, all

samples were diluted in saline and inoculated into TSB at a final concentration of ca. 5 x 10^6 CFU/ml. The inoculated tubes were then incubated separately at 55° (n=6), 60° (n=10), and 65°C (n=10) in a circulating water bath. The internal temperature of the broth was monitored by J-type thermocouples (DCC Corporation, Pennsauken, NJ) attached to a Hotmux data logger (DCC Corporation) and tubes were removed at pre-determined time intervals. After cooling in an ice bath, the inoculated tubes were then spiral plated onto TSA with a Autoplate 4000 (Spiral Biotech Norwood, MA) and incubated at 37°C for 24 h. The log CFU/ml values were plotted against heating time to determine the line of best fit. The formula for D-value calculation was $D = t / (\log N_o - \log N_f)$, where t is total time, N_o is the intitial bacterial concentration, N_f is the final bacterial concentration, and D is the D-value. D-values were determined for each isolate at the three tested temperatures. Z-values were determined by plotting log D-values (y-axis) vs. heating temperatures (x-axis). The z-value is the temperature change required to change the D-value by 1 log.

Serotyping and PFGE analysis of *Salmonella* isolates. Twenty-nine *Salmonella* isolates were serotyped by the FDA lab using the Kauffman-White Classification system, and the PFGE patterns of these isolates were analyzed following PulseNet's standard PFGE protocol for *Salmonella* (Ribot 2006). Briefly, plugs were prepared by mixing SeaKem Gold agarose and 1% sodium dodecyl sulfate (SDS) with *Salmonella* (10^8 CFU/ml) and proteinase K. Once cell lysis was complete, the plugs underwent restriction digestion by *Xba*I. The plugs were then washed, cast into an agarose gel, and underwent

electrophoresis using a CHEF Pulse-Field system (Bio-rad). The gel was stained with ethidium bromide and imaged using a Gel Doc system (Bio-Rad).

Statistical analysis. All plate count data were converted into $\log_{10}$ values. The mean counts and standard deviations were determined for the samples according to meal type as well as by the commercial renderer. The data were subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at $P = 0.05$. The Tukey's multiple comparison procedure of the Statistical Analysis System (SAS 2001, Cary, NC.) was used to compare all pairs of means when the test criterion for the analysis of variance was significant.

Results

In this study, 150 fresh samples of feather, meat, MBM, MBM-P, and blood meals were analyzed. Feather, meat, MBM, and poultry meals were provided by at least 4 companies whereas blood and MBM-P were produced by a single company.

The average pH of the meal types ranged from 6.16 to 7.36 (Table 2.1). The pH of blood meal was higher ($P < 0.05$) than those of all other meal types. Feather meal contained the highest amount of moisture (5.99%); whereas, meat meal contained the lowest amount of moisture (2.5%) (Table 2.1). Both feather and blood meals were higher ($P < 0.05$) in moisture content than meat and MBM meal. The water activity of different types of meal was analyzed; however, the a_w values in the range of 0.41 to 0.49 were consistent among the meal types (data not shown).

Total bacterial counts (TBCs) ranged from 1.70 to 6.68 log CFU/g, and enterococci counts ranged from less than 1.40 to 5.70 log CFU/g. The mean TBCs for

each meal type ranged from 3.70 to 5.88 log CFU/g; whereas, mean enterococci counts were in the range of 1.60 to 3.85 log CFU/g (Figure 2.1). Total bacterial counts were found to be the highest in blood meal and the lowest in meat meal. Enterococci counts were also the highest in blood meal but the lowest in the MBM-P. Blood meal was more contaminated ($P < 0.05$) with total bacteria than other meal types, and both blood and feather meals contained higher levels ($P < 0.05$) of enterococci.

Comparisons between meal type and company for TBCs and enterococci counts are presented in Tables 2.2 & 2.3, respectively. Since 38 out of 150 samples were received without the company information, those samples were excluded in both Tables. There were differences ($P < 0.05$) in TBCs for feather, meat, MBM, and poultry meal types among companies. The feather and poultry meal produced by company F were contaminated with the highest ($P<0.05$) levels of total bacteria for that meal type. There were also differences ($P < 0.05$) in enterococci counts in MBM and poultry meal among companies. All meals (feather, meat, or poultry) produced by company A and F had the highest levels ($P < 0.05$) of enterococci contamination. For company H, blood meals contained higher TBC and higher enterococi counts than the other four meal types they produce. *Enterococcus* spp. was detected in 122 of 150 samples (81.3%), and accounted for up to 54% of the total bacterial counts in some samples (Table 2.4). Over 90% of the blood or feather meals were positive for enterococci.

E. coli was not detected in any of the samples tested, but coliforms were detected in a few samples (4 out of 150) of the feather, MBM, and poultry meals (Table 2.4). *Salmonella* was detected in 13 of the 150 samples (8.7%) including feather, meat, MBM,

and poultry meals. In general, the poultry meal samples had the highest contamination rate (13.7%) for *Salmonella* (Table 2.4).

Approximately 76% of the 200 isolates collected were confirmed enterococcus by traditional confirmational tests. Vancomycin resistance screening revelaed that of the 152 enterococcus isolates only 16 (10.5%) are potentially resistant. These 16 isolates were confirmed as enterococcus by PCR assay. PCR analysis also indicated none of the 16 isolates were *E. faecalis* or *E. faecium* (data not shown). The results of the agar dilution test revealed that the MIC of 13 enterococci isolates was 16 µg/ml or less; whereas, only three isolate were able to grow at 32 µg/ml. Since the breakpoint for VRE is 32 µg/ml, three (2%) enterococci isolates were considered VRE in this study (Willey 1992). PCR analysis of the 16 isolates further revealed that none of the isolates contained the *vanA* or *vanB* that are traditionally associated with higher levels of vancomycin resistance.

After initial thermal resistance screening, six *Salmonella* isolates were tested further for D- and z-vlaue determinations. Of those six isolates, five were isolated from the rendered products consisting of different serotypes as well as an additional isolate provided by SC DHEC from a SC boiled peanut outbreak were used to determine D- and z-values (Table 2.5). There was no difference ($P < 0.05$) between all determined D-values at each temperature with a maximum range of 0.85 minutes between them. All z-values were approximately 7°C.

A total 13 distinguishable serotypes including Senftenberg, Oranienburg, Idikan, Johannesburg, IIIa. 42: z4,z23, Banana, Demerara, Putten, Molade, Montevideo, Mbandaka, Livingstone, and Amsterdam were identified. PFGE analysis was able to

further classify all distinguishable serotypes except those that were *S.* Banana. There were 16 PFGE patterns for 12 serotypes, including three types of *S.* Senftenberg, two types of *S.* Mbandaka, and two types of *S.* Demarara.

Discussion

To ensure the microbiological safety of rendering end products, rendering companies use various forms of heat treatment depending on the meal type. Three forms of heat treatment were analyzed in this study. The first method of treatment is for meat, MBM, MBM-P and poultry meal. This process uses a continuous rendering process, which heats the raw material in a horizontal, steam-jacketed cylindrical vessel to a final temperature ranging from 121 to 135°C (Franco and Swanson 1996). After the fat is removed, the meal is dried and packaged (National 2005). In the continuous process of blood meal, the whole blood is passed through a system of tubes where steam is injected to coagulate blood solids. After separation, the blood solids are fed into a gas-fired drying system, direct-contact ring dryer, a steam tube, or rotary dryer (Franco and Swanson 1996). As for feather meal production, the keratin of poultry feathers is first converted into short chain amino acids by chemical hydrolysis in a batch cooker at temperatures ranging from 138 to 149^{o}C under pressure. The feather meal is then dried in a manner similar to the way blood meal is (McGovern 2000). In this study, total bacterial and enterococci counts based on meal type from the highest to lowest were: blood meal > feather meal > meat meal. This would suggest that the continuous rendering system for meat, MBM, and poultry meal is more effective than the methods used for feather and blood meal in eliminating bacteria. Although blood meal samples

were from a single company (H), both total bacterial and enterococci counts in blood meal were higher ($P<0.05$) than those in MBM, poultry, and meat meal produced by this company. In addition, the microbial contamination levels of raw materials can also affect the bacterial levels and contents in the finished meal products. A 2001 study indicated that raw material used for rendered by-product production contains the following levels of pathogens: *Salmonella* spp. 84.5%, *Clostridium perfringens* 71.4%, *Listeria monocytogenes* 8.3%, *Listeria* spp. 76.2%, *Campylobacter jejuni* 20.0%, and *Campylobacter* spp. 29.8% (Troutt et al. 2001).

Moisture is one of the key control points for preventing microbial growth of the rendered product. In both blood and feather meal processing, the moist meals with ca. 50 to 55% moisture are subjected to drying If these meals are not properly dried, the moisture content will remain high and microbial levels may increase as evidenced in our study for both blood and feather meals. Although the moisture levels in animal meals analyzed in this study were much lower than the minimal moisture level for bacteria growth, some bacteria esp. *Salmonella,* if present, may persist for extended period of time during storage (Doyle et al., 2001;Meat 1997).

The machinery, plant environment and workers can all serve as possible sources of contamination as well. It is possible for bacteria to form biofilm on the machinery, conveying belts, containers, and floor of the processing plant. Foodborne pathogens such as *E. coli* O157:H7, *Listeria monocytogenes, Yersinia enterocolitica,* and *Campylobacter jejuni* form biofilms on food surfaces and food contact equipment (Chae and Schraft 2000, Doyle et al. 2001, Kumar et al. 1998). Recontamination can also occur in finished

meals during storage within the plant. Therefore, it is important to analyze the effect that the practices of various companies had on the contamination levels of the samples. Tables 2.2 and 2.3 revealed that all meals produced by company A and F contained higher ($P < 0.05$) levels of enterococci and total bacteria. Although there were some significant differences among the companies, it is not possible to discern if one company was any less contaminated than the others due to unequal numbers of meal types provided by each company.

Rendered animal products are frequently contaminated with *Salmonella*. Epidemiological studies also have linked human *Salmonella* spp. to contaminated animal feed (Crump et al. 2002). For example, a *S. enterica* serotype Heidelberg outbreak in 1963 led to 77 infections from milk consumption (Knox et al. 1963). This outbreak was linked to bovine mastitis caused by the same *Salmonella* serotype, which was found in the meat and bone meal fed to the cows. Over a four month period, *Salmonella* was detected in 81% of the meat meal, 40% of the feather meal, and 84.2% of the broiler premix (meat meal as a filler) produced in Ontario feed mills (Hacking et al. 1977). Loken et al. (1968) tested 1,395 rendered products from seven different plants and detected the presence of *Salmonella* in 241 (17%) of the samples. The study also tested the plant with environmental swabs, and *Salmonella* was isolated from 359/1901 (19%) of the swabs. In 1993, a FDA study of feed samples from 78 protein-based rendering plants detected *Salmonella enterica* in 56% of the 101 samples collected (McChesney et al. 1995). A year later a FDA test of 89 samples detected *S. enterica* in 25% of the samples (Crump et al. 2002). Members of the Animal Protein Producers Industry (APPI)

tested samples weekly for *Salmonella* for at least 52 tests a year (Franco 2005). On average, twenty-five percent of the samples tested positive for *Salmonella*. Results from 197 *Salmonella*-positive samples during a particular year indicated that the average MPN/g was 16.3 with a range of 0.2 to 78.0 MPN/g (Franco 2005). Of the *Salmonella* serovars found in that study, four were associated with food borne illness (*S.* Typhimurium, *S.* Enteritidis, *S.* Agona, and *S.* Infantis). Unlike samples analyzed by FDA studies, which had been transported and subjected to secondary contamination, the present study analyzed the rendered animal products collected at the end of the processing line. As compared with above surveys, the low contamination rate for *Salmonella* (8.6%) and absence of *E. coli* in 150 meal samples analyzed may suggest that the rendering industry has made progress toward improving the safety of animal co-products. In addition, samples positive for *Salmonella* were mostly poultry meal or feather meal. A possible explanation is that the raw poultry products may contain larger numbers of *Salmonella* entering the rendering plants which would require additional heat exposure compared to the other meal types. Therefore, the initial microbial load of raw materials should be considered in order to produce microbiological safe meal products. The overall goal set forth by the FDA Center for Veterinary Medicine in 1990 is for both animal feed ingredients and the final feed to contain no detectable amount of *Salmonella*, zero tolerance (Meeker 2006).

Cross-contamination may be one of the major reasons for the presence of *Salmonella* in the finished products. In this study, all *Salmonella* isolates had D-values in the range of 9 minutes at 55°C, 2 minutes at 60°C, and under 45 seconds at 65°C. The

temperatures produce in the batch cookers used for meal processing reaches 121-135°C and are maintained for 2 to 3 h, which far exceeds the maximum temperature used in this study. At 65°C all isolates in this study were reduced by 1 log within 45 seconds indicating that the rendering process should be sufficient in killing all *Salmonella* provided that the internal temperature of the by-products reaches or exceeds the cooking temperature. D-values for *Salmonella* were reported as 18-594 min at 50°C in corn flour (van Cauwenberged et al., 1981), 55-345 min at 70°C in wheat flour (Archer at al., 1998), and 10-115 min at 70°C in meat and bone meal (Liu et al., 1969). Apparently, the D-values of the *Salmonella* in this study are lower than those reported for *Salmonella* in above dry materials, suggesting that the *Salmonella* contamination in rendered animal meals is most likely a result of post-processing contamination. The PFGE was used to characterize the *Salmonella* isolates within the processing plant. A comparison between each individual set of PFGE types and what type of product that were isolated from, where they were collected, and when they were collected revealed that there was not a single type present in a particular plant environment over an extended period of time. This indicates that the likely source of contamination is the raw animal by-products due to the fact it is a consistent incoming new source for contamination.

Enterococcus spp. is widely distributed in the environment and animals. Enterococci also have been consistently isolated from the carcass of beef, poultry, and pork meat (Franz et al. 2003). Knudtson and Hartman (1993) reported that pig carcasses from three abattoirs were contaminated with enterococci at 4~8 log CFU per 100 cm^2 of the carcass surface, with *E. faecium* and *E. faecalis* as the dominant species. Enterococci

being found in rendered meals may be less surprising than other bacterial contamination due to the fact that enterococci are heat tolerant. A few studies previously reported that *Enterococcus* spp. was detected in rendered animal meals (Cullen et al. 2003, and Schwalbe et al. 1999). A 2003 national survey conducted by the FDA was performed on 122 samples (MBM 72, Blood 16, Bone 2, Feather 10, Poultry 17, and Fish 5) (Cullen et al. 2003). Of these, 84% of the samples contained *Enterococcus* spp. which were identified as *E. faecium* (86.5%), *E. faecalis* (7.5%), *E. gallinarum* (2%), *E. hirae* (*1.5%),* and *E. avium* (0.5%). The present study revealed a similar contamination rate by enterococci (81.3%) with a range from below the detection limit (<1.40 log CFU/g) to 5.70 log CFU/g. MIC results revealed that VRE was present in the rendered products but with low prevalence (2%). In addition, none of the VRE isolates were *E. faecalis* or *E. faecium.* The *vanA* and *vanB* genes confer high levels of resistance (≥64µg/ml) and are the only two resistance genes that have been associated with plasmids. Due to the low MIC for vancomycin, the VRE isolates were assumed not to posses the *vanA* or *vanB* genes, suggesting a low possibility that there is transference of the vancomycin resistance genes between other species found in the rendered meal (Gilmore 2002). The absence of the *vanA* and *vanB* gene was then confirmed by PCR analysis.

This study has provided some baseline data on microbiological contamination levels in fresh rendered meals. These results more accurately represent the microbial load in rendered products than previous studies since all samples were taken directly from the production lines before the rendered meals could be mixed with any other feed components in a feed mill. Although avoiding the contamination of finished animal

meals is difficult in post-production shipment and reformulation, using heat tolerant enterococci as a marker for bacterial load may help the industry to determine if the meal products have been heated adequately or have been contaminated by raw materials or the processing environment. Based on the low thermal resistance of *Salmonella* isolates tested in this study, the heat treatment process used by the rendering industry appears to be adequate in eliminating bacterial contamination in the product; therefore the *Salmonella* contamination is most likely resulting from post-processing contamination. Overall, the low prevalence of *Salmonella* contamination and absence of *E. coli* in all samples indicate that the rendering industry has microbiologically improved the products.

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Figure Legend

Fig.2.1: Total bacterial and enterococci counts for six meal types. Reported values are expressed as the mean ± standard deviation. The different letters above each bar are used to indicate the significant ($P < 0.05$) differences between the counts of the meal types.

Table 2.1. Moisture content and pH level per meal type

Meal Type	Moisture % (mean ± std dev)	pH (mean ± std dev)
Feather (n=30)	$5.99 \pm 2.48^{A*}$	6.20 ± 0.39^{B}
Meat (n=14)	2.50 ± 1.49^{C}	6.38 ± 0.58^{B}
MBM (n=37)	2.90 ± 1.23^{C}	6.16 ± 0.52^{B}
Poultry (n=51)	3.21 ± 1.15^{BC}	6.41 ± 0.28^{B}
Blood (n=12)	4.94 ± 2.17^{AB}	7.36 ± 1.08^{A}
MBM-P (n=6)	4.04 ± 2.41^{BC}	6.37 ± 0.29^{B}

**Means with different superscripts are significantly different ($P < 0.05$) among meal types.*

Table 2.2. Total bacterial counts of rendered animal meals produced by 12 companies

Company	Total bacterial counts (log CFU/g)					
	Feather (n=30)	Meat (n=14)	MBM (n=37)	MBM-P (n=6)	Poultry (n=51)	Blood (n=12)
A	$4.54 \pm 0.71^{AB*}$	4.78 ± 0.62^{A}	N/A	N/A	4.59 ± 0.87^{AB}	N/A
B	3.68 ± 0.26^{B}	4.80 ± 0.62^{A}	N/A	N/A	4.52 ± 0.65^{AB}	N/A
C	N/A^{**}	N/A	N/A	3.98 ± 0.64	N/A	N/A
D	N/A	2.61 ± 0.46^{B}	5.04 ± 0.64^{A}	N/A	N/A	N/A
E	N/A	N/A	4.76 ± 0.69^{A}	N/A	N/A	N/A
F	5.38 ± 0.86^{A}	N/A	N/A	N/A	5.52 ± 0.38^{A}	N/A
G	N/A	N/A	4.61 ± 0.34^{A}	N/A	N/A	N/A
H	4.65 ± 0.52^{AB}	3.71 ± 0.90^{AB}	4.11 ± 0.70^{AB}	N/A	3.86 ± 0.83^{B}	5.91 ± 0.79
I	N/A	N/A	3.98 ± 0.69^{AB}	N/A	N/A	N/A
J	N/A	N/A	2.20 ± 0.38^{B}	N/A	N/A	N/A
K	N/A	3.99 ± 0.21^{AB}	5.04 ± 1.09^{A}	N/A	N/A	N/A
L	N/A	N/A	4.24 ± 0.94^{AB}	N/A	N/A	N/A

**The different letters in each column are used to indicate the significant differences (P<0.05) between the companies for that given meal type.*

***N/A means that there were no samples of that meal type from the corresponding company*

Table 2.3. Enterococci counts of rendered animal meals produced by 12 companies

Company	Enterococci counts (log CFU/g)					
	Feather (n=30)	Meat (n=14)	MBM (n=37)	MBM-P (n=6)	Poultry (n=51)	Blood (n=12)
A	$2.94 \pm 0.78^{A*}$	3.16 ± 0.45^{A}	N/A	N/A	2.27 ± 1.26^{AB}	N/A
B	2.38 ± 0.38^{A}	3.10 ± 0.45^{A}	N/A	N/A	2.95 ± 0.76^{A}	N/A
C	N/A**	N/A	N/A	1.15 ± 1.03	N/A	N/A
D	N/A	0.57 ± 0.84^{A}	2.18 ± 0.90^{AB}	N/A	N/A	N/A
E	N/A	N/A	2.32 ± 1.10^{AB}	N/A	N/A	N/A
F	2.83 ± 1.61^{A}	N/A	N/A	N/A	3.35 ± 0.07^{A}	N/A
G	N/A	N/A	2.88 ± 0.20^{A}	N/A	N/A	N/A
H	2.58 ± 1.06^{A}	1.78 ± 1.51^{A}	0.97 ± 1.41^{AB}	N/A	1.16 ± 1.38^{B}	2.97 ± 1.53
I	N/A	N/A	1.57 ± 1.38^{AB}	N/A	N/A	N/A
J	N/A	N/A	BLD^{B}	N/A	N/A	N/A

K	N/A	BLD[A***]	3.21 ± 0.80[A]	N/A	N/A	N/A
L	N/A	N/A	2.63 ± 1.33[AB]	N/A	N/A	N/A

** Means with different superscripts are significantly different (P < 0.05) between the companies for that given meal type.*

***N/A means that there were no samples of that meal type from the corresponding company.*

****BLD, Below the minimum detection limit (100 CFU/g).*

Table 2.4. Percentages of samples of each meal type that was positive for *Salmonella*, enterococci, and coliforms

	% *Salmonella* (n)	% enterococci (n)	% coliforms (n)
Feather (n=30)	6.7(2)	96.7(29)	6.7(2)
Meat (n=14)	7.1(1)	64.3(9)	0(0)
MBM (n=37)	8.1(3)	75.7(28)	2.7(1)
Poultry (n=51)	13.7(7)	78.4(40)	2.0(1)
Blood (n=12)	0(0)	91.7(11)	0(0)
MBM-P (n=6)	0(0)	83.3(5)	0(0)
Total (n=150)	8.7(13)	81.3(122)	2.7(4)

1

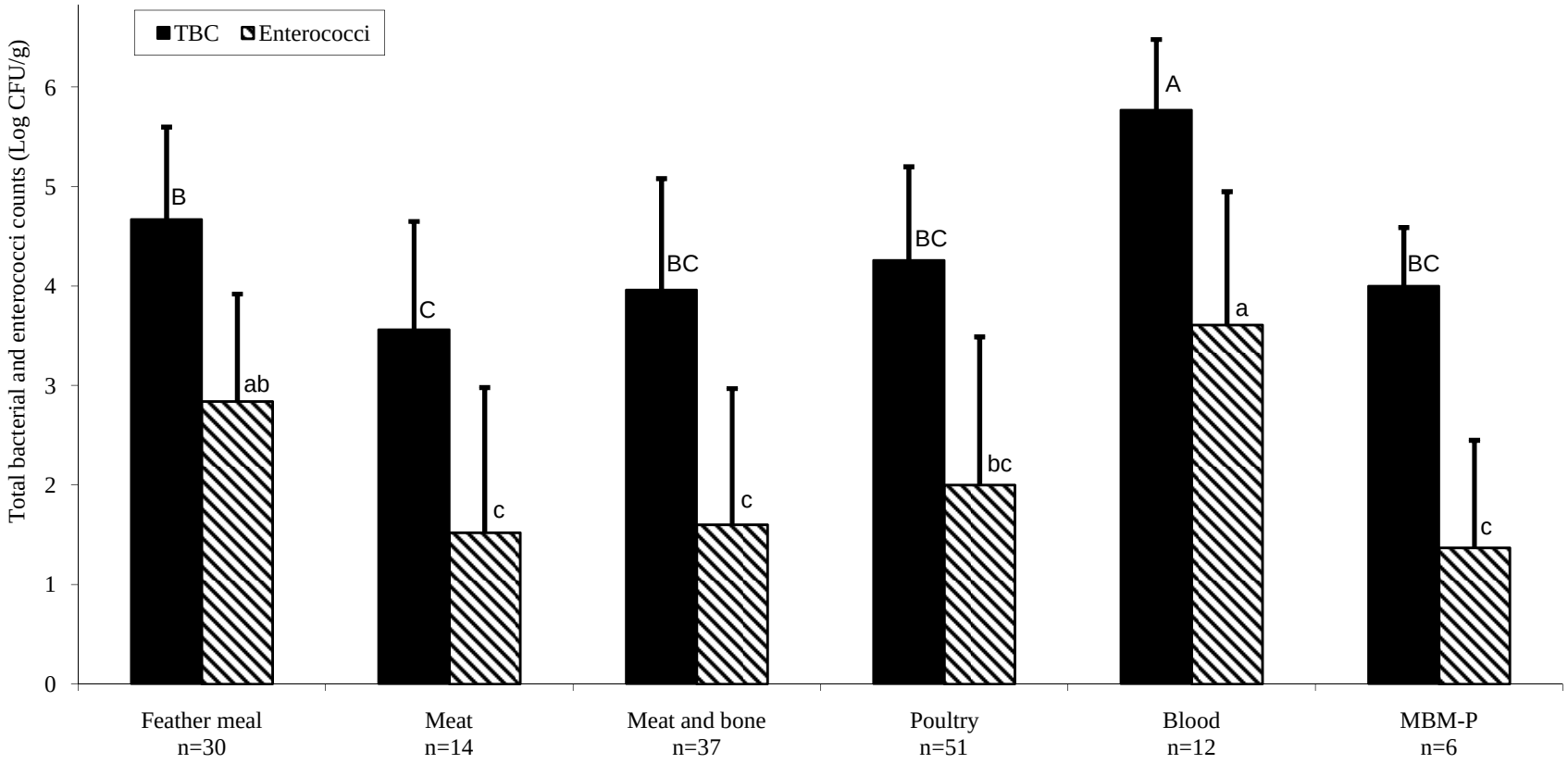
Table 2.5. D-values and z-values of *Salmonella* Isolates

Isolate	Serotype	D-value (min) at given temperature			z-value (°C)
		55°C	60°C	65°C	
B111419*	Enteritidis	10.12 ± 0.62^{A}	2.47 ± 0.64^{A}	0.50 ± 0.12^{A}	7.66
23B2	Senftenberg	9.27 ± 0.43^{A}	2.07 ± 0.28^{A}	0.36 ± 0.18^{A}	7.09
98	Idikan	9.30 ± 1.66^{A}	2.28 ± 0.53^{A}	0.36 ± 0.21^{A}	7.08
214	Mbandaka	9.76 ± 1.43^{A}	2.19 ± 0.71^{A}	0.43 ± 0.13^{A}	7.37
222	Banana	9.47 ± 0.82^{A}	2.08 ± 1.03^{A}	0.35 ± 0.17^{A}	6.98
223B	Demerara	9.99 ± 0.42^{A}	2.15 ± 1.02^{A}	0.40 ± 0.14^{A}	7.16

1 ** Isolate provided by SC DHEC*

2 ******* *Means with different superscripts are significantly different (P < 0.05)*

Figure 2.1


TBC
Enterococci
Total bacterial and enterococci counts (Log CFU/g)
0
1
2
3
4
5
6
B
ab
C
c
BC
c
BC
bc
A
a
BC
c
Feather meal
n=30
Meat
n=14
Meat and bone
n=37
Poultry
n=51
Blood
n=12
MBM-P
n=6

CHAPTER THREE

REDUCTION OF*SALMONELLA* ON ENVIRONMENTAL SURFACES WITH BACTERIOPHAGE TREATMENT

Abstract

A previous study has suggested that the presence of *Salmonella* in animal meal is due to post processing contamination. The objective of this study was to determine if a bacteriophage cocktail could be used to reduce the risk of cross-contamination by reducing *Salmonella* levels on environmental surfaces found within a rendering facility. A cocktail of five bacteriophages was isolated and purified for two dominant *Salmonella* serotypes (Enteritidis and Typhimurium) and three other serotypes (Mbandaka, Johannesburg, and Idikan) that were previously isolated from finished rendered products. These *Salmonella* serotypes were treated with the bacteriophage cocktail at varying concentrations to determine the optimal multiplicity of infection (MOI). The optimized cocktail was then used to treat the attached *Salmonella* cocktail and *S.* Enteritidis within a single and double species biofilm on templates of steel, plastic, cement and rubber at 20°, 30°, and 40°C. The bacteriophage cocktail was found to be most effective at a MOI of 10. Bacteriophage treatment of the surface materials with attached *Salmonella* resulted in a ca. 2 log decrease in the *Salmonella* count at 40° and 30°C, and ca. 1 log CFU/cm^2 decrease at 20°C for all surface materials. Bacteriophage treatment of the *S.* Enteritidis single-species biofilm resulted in a ca. 1-2, 2-3, and 1 log CFU/cm^2 reduction in *Salmonella* populations at 20, 30, and 40°C, respectively. Bacteriophage treatment of the double species biofilm at 20, 30, and 40°C resulted in reduction of the *Salmonella* populations by ca. 0.5, 1.5-2.0, and 0.5 log CFU/cm^2. Overall, the bacteriophage treatment was most effective at 30°C under all test conditions. These results demonstrated that a bacteriophage cocktail significantly reduces levels

of *Salmonella* contamination on environmental surfaces, and may potentially decrease the incidence of cross-contamination in rendering facilities.

Introduction

Annually, the U.S. rendering industry produces ca. 11.2 billion pounds of protein and 10.9 billion pounds of fats. About 85% of these products are then used in the production of animal feed ingredients (Meeker and Hamilton, 2006). While rendered by-products have many uses, the primary usage is as an additive to animal feed. The contamination of the feed can serve as a vehicle for pathogenic bacteria, such as *Salmonella*, to enter the food chain (Crump et al., 2003; Knox et al., 1963). This was demonstrated by a *S.* Heidelberg outbreak in 1963 that was traced back to milk produced from cows infected with the serotype. Microbiological analysis revealed that the source of the infection in the cattle was the meat and bone meal used as an additive in the feed (Knox et al., 1963).

The presence of *Salmonella* has been documented in finished rendered by-products as well as in enivornmental swab samples from the rendering facilities (Kinley et al., 2009; Hofacre et al., 2001; Loken et al., 1968). Troutt et al. (2001) detected 12 different serotypes of *Salmonella* spp. in raw materials derived from animal production and processing, materials from the cooking/expelling process, and in final rendered products. Recently, in an analysis of ca. 200 rendered animal products collected from 12 processing plants in the U.S., we detected 13 *Salmonella* serotypes with an overall contamination rate of 8.7% (Kinley et al., 2009). Four serotypes, Johannesburg, Oranienburg, Putten, and Senftenberg, detected matched those reported by Troutt et al. (2001).

It is likely that the presence of *Salmonella* in rendered animal by-products is due to post-processing contamination (Kinley, 2009). To prevent cross-contamination by *Salmonella* in the

rendering environment, the most commonly used treatments on surfaces include using chemical sanitizers (bleach and trisodium phosphate) or in rendered animal meal by adding chemical or biological feed additives such as Sal Curb®, propionic acid, sorbic acid, acetic acid, and benzoic acid (Larsen et al., 1993).

Bacteriophages, viruses of specific bacterial hosts, have been used as a biological control method for various applications. Several studies have demonstrated that bacteriophage treatment can be very effective against *Salmonella* contamination. Modi et al. (2001) demonstrated that the addition of bacteriophage to a cheddar cheese starter culture significantly reduced the risk of contamination in the final product. Whichard et al. (2003) reported that Felix 01 bacteriophage can be used to control *S.* Typhimurium contamination on chicken frankfurters. A study by Leverentz et al. (2001) reported that bacteriophages reduced *Salmonella* contamination on fresh cut honey-dew melon by 3.5 logs at 5°C and 10°C, and a 2.5 log reduction at 20°C. Additionally, the simultaneous use of two different bacteriophages against three *Salmonella* serotypes has been shown to reduce *Salmonella* populations in sprout seeds by ca. 0.5 log (Pao, 2004). Currently, there is a lack of information on bacteriophage application in rendering environments or in finished animal by-products.

The purpose of this study was to develop a bacteriophage cocktail capable of effectively reducing *Salmonella* contamination on environmental surfaces found within the rendering plant environment.

Materials and Methods

Bacteriophage isolation. Bacteriophages were isolated from raw chicken offal using 12 *Salmonella* serotypes (Senftenberg, Oranienburg, Idikan, Johannesburg, IIIa. 42: z4, z23, Banana, Demerara, Putten, Molade, Montevideo, Mbandaka, Livingstone, and Amsterdam)

previously isolated from finished rendered by-products as host strains. Each individual culture was mixed with the raw material and double strength tryptic soy broth (DS-TSB, Becton-Dickinson and company, Sparks, MD), and incubated for 24 h at 37°C. The culture was then centrifuged and the supernatant was filtered using a 0.2 µm syringe filter (VWR). The supernatant was spot plated onto tryptic soy agar (TSA, Becton-Dickinson and Company) with an overlay containing the respective *Salmonella* serotype used to propagate the bacteriophage. The resulting plaques were removed using a sterile 1 ml pipette and suspended in sterile SM buffer (5.8g l^{-1} NaCl, 2g l^{-1} $MgSO_4$*$7H_20$, and 1M pH 2.5 50ml l^{-1} Tris-HCl). The bacteriophages were purified by repeating the process of suspending the agar plugs in sterile SM buffer for 1 h and replating on a TSA plate overlayed with the respective *Salmonella* serotype twice. After purification, the resulting plaques were suspended in SM buffer and incubated at 4°C for 24 h. Following incubation, the suspension was removed with a sterile pipette and syringe filtered (0.2 µm). The resulting bacteriophage suspension was stored at 4°C and used to produce stocks.

Bacteriophage FO was kindly provided by the University of Calgary and a stock solution was prepared as described above. The host strain for this bacteriophage during this study was *S.* Idikan.

Bacteriophage preparation. The host strain for each bacteriophage was grown in tryptic soy broth (TSB) overnight, and one milliliter of the culture was inoculated into 250 ml of fresh TSB. The bacteriophage was added to the TSB at a multiplicity of infection (MOI) of 0.1, and the suspensions were incubated with agitation (3 rpm) at 37°C for ca. 4-5 h. Chloroform was added to the suspensions, and then after incubation at 37°C for 10 min, the suspension was vacuum filtered using a disposable water filtration unit (Nalgene, 0.45µm). The filtered solution was centrifuged at 70,000 x g for 90 min. The pellet was resuspended in 5 ml of SM buffer, and

incubated at 37°C for 1 h. The filtered bacteriophage solution yielded a titre of 10^{10} to 10^{12} PFU/ml.

Transmission electron microscopy: Copper grids (400 mesh, EMS, Hatfield, PA) were coated with 0.5% formvar dissolved in dichloroethane (Fischer, Fairlong, NJ). Dried grids were placed on parafilm (American National Can, Menasha, WI) and 5 µl of phage solution (10^{10} PFU/ml) was pipetted on the surface for 1 min. The liquid was drawn off with filter paper and the grid was stained for 30 seconds with 5 µl of 0.5% phosphotungstic acid (PTA; EMS, Hatfield, PA) or 2% uranyl acetate (UA; EMS, PA). Grids were air-dried and viewed on a Hitachi H-7600 electron microscope (Hitachi, Tokyo, Japan) at 120-keV accelerating voltage.

Bacteriophage cocktail optimization. Two commonly isolated *Salmonella* serotypes (Typhimurium and Enteritidis) and three previously isolated serotypes from rendered products (Idikan, Mbandaka, and Johannesburg) were used for this study. For each of these isolates, at least one bacteriophage was selected based on a series of host range testing. Briefly, all bacteriophages were spotted onto TSA overlayed with every *Salmonella* serotype being tested. The lytic activity of each bacteriophage was determined by the amount of lysis observed on the plate. Before combining the bacteriophage isolates into a cocktail, the MOI needed for complete lysis was determined for each bacteriophage individually by plating a series of dilutions of each bacteriophage onto a TSA plate overlayed with its individual targeted *Salmonella* serotype. The lowest concentration of bacteriophage that resulted in complete lysis was then used to compose the bacteriophage cocktail.

To determine if the cocktail was effective, each *Salmonella* serotype was diluted in TSB and transferred to a 96-well microplate. SM buffer was added to the control group and each bacteriophage was added to the serotype it was selected for at the previously determined

concentration. The microplate was incubated at room temperature and O.D. readings were determined at 600 nm using a µQuant™ spectrophotometer (Bio-Tek Winooski, VT) at set time intervals. This procedure was carried out on each serotype and a mixture of the serotypes using the bacteriophage cocktail at MOIs of 1, 10, and 50.

Attaching *Salmonella* to environmental surface materials. Stainless steel, concrete, high density polyethylene (HDPE) plastic, and rubber were cut into 25 cm^2 templates. The stainless steel templates were washed with acetone, etched by submerging the templates in 5N HCl for 15 min, washed with Micro-90® (International Products Association Burlingotn, NJ) detergent, and rinsed with sterile nanopure water (Joseph et al., 2001). The concrete templates were cleaned using a scrubbing brush and rinsed with sterile nanopure water. Both HDPE plastic and rubber templates were washed with a detergent and rinsed with sterile nanopure water.

Each dry template was inoculated with the mixture of 5 *Salmonella* serotypes by spreading the suspension over the entire template surface using a sterile inoculating loop to yield an initial concentration of ca. 10^5 CFU/cm^2. The templates were allowed to air-dry for ca. 6 h at room temperature inside of a purifier class II biosafety cabinet (Labconco Kansas City, MO). To remove unattached cells, templates were washed once with 10 ml sterile phosphate buffered saline (PBS). The templates were transferred to petri dishes containing a mixture of TSB and SM buffer using sterile forceps.

The effect of nutrient debris on surfaces with attached bacterial cells was also studied. The *Salmonella* cocktail was mixed into the liquefied raw material (raw chicken offal mixed and ground with sterile saline 1:1), spread over the templates, and air-dried to yield an initial concentration of 10^5 CFU/cm^2, as described above.

Biofilm formation on the environmental surfaces. A green fluorescent protein (gfp) labeled *S.* Enteritidis H4717 was used for biofilm studies. This organism contains a plasmid encoded with ampicillin resistance 100µg/ml. To produce a single species biofilm, the templates were submerged in 30 ml of 20% TSB with ampicillin (100 µg/ml) that was inoculated with the *S.* Enteritidis at a concentration of 10^5 CFU/ml and incubated at 30°C for 6 days. The templates were removed every other day, washed with sterile PBS, and placed back into fresh 20% TSB. To produce the multiple species biofilm, the templates were submerged in 30 ml of 20% TSB, which was inoculated with the gfp-labeled *S.* Enteritidis H4717 and *Pseudomonas aeruginosa* at a concentration of 10^5 CFU/ml for each culture, and incubated at 30°C for 6 days. The templates were handled in the same manner as those with the single species biofilm.

Bacteriophage treatment of artificially contaminated environmental surfaces. For bacteriophage treatment of templates with attached cells, the templates were suspended in the mixture of SM buffer and TSB inside a petri dish. The bacteriophage cocktail was added to the solution at a final concentration of 10^6 PFU/ml. The control templates were treated with SM buffer instead of the bacteriophage cocktail. The templates were incubated at 20, 30, and 40°C, and removed at pre-determined time intervals. At each sampling time, the surfaces of the templates were swabbed thoroughly using a sterile cotton swab, and then transferred into sterile saline. The suspension was centrifuged for 10 min at 5,100 rpm to separate bacterial cells from remaining bacteriophage particles. The supernatant was decanted and bacterial cells were resuspended and serially diluted with sterile saline. All dilutions were spiral-plated onto TSA with an Autoplate 4000 (Spiral Biotech Norwood, MA), and incubated at 37°C for 24 h.

Biofilm templates were analyzed using the same procedure as described above, with one exception. After the addition of the bacteriophage cocktail, the templates were either incubated at

37°C for 15 min to produce biofilms with absorbed bacteriophage, or the templates were directly incubated at the test temperatures (unabsorbed bacteriophage). *Salmonella* populations were determined as described above. When analyzing the multiple species biofilm containing *P. aeruginosa*, *Salmonella* was enumerated with xylose lysine tergitol-4 plates (XLT-4, Becton-Dickinson and company).

Confocal imaging of the surfaces with single and multi-species biofilms treated with bacteriophages. After bacteriophage treatment at 30°C, templates containing biofilm were viewed using a confocal scanning laser microscope (LSM 510, Karl Zeiss, Germany) equipped with an argon laser (458, 477, 488, and 514 nm) and two HeNe lasers (543 and 633 nm) along with untreated templates to visualize the effect the bacteriophage had on the biofilm. The PMT1 emmision filters were set at band pass (BP) of 505-520 nm and long pass filter (LP) of 580 nm to detect the gfp-labeled *Salmonella* cells.

Chemical treatment of environmental surfaces artificially contaminated with *Salmonella*. For comparative purposes, two chemical sanitizers were used to treat the environmental surfaces containing the attached *Salmonella,* attached *Salmonella* with organic material, single-species biofilm, and double-species biofilm. The two chemicals were bleach solution (Clorox®), containing 200 ppm sodium hypochlorite, and 12% trisodium phosphate (TSP). The treatment procedure was performed as described for the bacteriophage treatment, except chemical sanitizers were substituted for the bacteriophage cocktail.

Statistical analysis. All plate count data were converted into log values. The mean *Salmonella* counts and standard deviations were determined for control and experimental samples for each method of treatment at all temperatures. The data were subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at $P = 0.05$. The Tukey's

multiple comparison procedure of the Statistical Analysis System (SAS 2001, Cary, NC.) was used to compare all pairs of means when the test criterion for the analysis of variance was significant.

Results

Bacteriophages were isolated from raw chicken offal for every *Salmonella* serotype used (n=12).. The number of bacteriophages isolated for each serotype is shown in Table 1. Two of those bacteriophages (JC1 and MbE2) were selected to produce a cocktail based on their lytic activity against *S.* Typhimurium, *S.* Enteritidis, *S.* Idikan, *S.* Mbandaka, and *S.* Johannesburg. The other three bacteriophages included 2 previously isolated from raw sewage (H3353S6p2 and H4717S5p2) and the FO bacteriophage. The TEM images of 4 of the bacteriophages (excluding FO) are presented in Fig. 1. The presence of a contractile tail for M226 and H4717S5p2 suggest that they belong to the family of *Myoviridae*. Bacteriophage J161 and H3353S6p2 had flexible non-contractile tails and were of the family *Siphoviridae*.

The MOI optimization study indicated that increasing the MOI from 1 to 10 enhanced the lytic activity of the bacteriophage; however, a MOI of 50 did not increase ($P>0.05$) the efficacy of the bacteriophage cocktail (Fig. 3.2a). MOI optimization also revealed that the cocktail's lytic activity was only sustained for a 12 h period. The addition of a different bacteriophage cocktail with a MOI of 10 was shown to extend the lytic activity for an additional 12 h (Fig. 3.2b).

An initial trial was conducted to determine the effect of residual bacteriophage particles on *Salmonella* enumeration following bacteriophage treatment. Following treatment on stainless steel at 30°C, *Salmonella* populations were compared when the sample suspension was directly plated with when it was centrifuged to remove the remaining bacteriophage. The results (Table 3.2) showed an insignificant difference ($P>0.05$) between the two sampling procedures; however,

to ensure there was no prolonged effect, samples collected during the current study were centrifuged prior to plating for *Salmonella* enumeration.

The bacteriophage cocktail reduced levels of the attached *Salmonella* on all surface material at all tested temperatures (Fig. 3.3). Bacteriophage treatment reduced *Salmonella* levels by ca. 1.0, 2.0-2.5, and 1.5-2.0 log CFU/cm^2 at 20, 30, and 40°C, respectively, within 6 h for all templates. The level of reduction was significant ($P<0.05$) for all temperatures on all surfaces (Table 3.3). The presence of an organic layer had no observed effect on the efficacy of the bacteriophage cocktail (Table 3.3). *Salmonella* reduction within the organic layer when treated with the bacteriophage was also significant ($P<0.05$) at all temperatures and on all surfaces.

The bacteriophage cocktail was also capable of reducing the amount of *Salmonella* Enteritidis H4717-gfp within an attached biofilm on all surfaces at the tested temperatures (Table 3.3). GFP-labeled *S.* Enteritidis H4717 was reduced by ca. 1.5-2.0, 2.0-4.0, and 1.0-1.5 log CFU/cm^2 at 20, 30, and 40°C respectively for all templates. As observed with the attached cells, the bacteriophage cocktail reduced *Salmonella* levels ($P<0.05$) in biofilm at the tested temperatures. Allowing absorption into the biofilm prior to incubation had no observable impact on the efficacy of the bacteriophage (data not shown). The effect of the bacteriophage cocktail on *S.* Enteritidis H4717-gfp within the biofilm can also be observed in the confocal images (Figure 3.4). On the treated slide (Fig. 3.4(c)) there is less gfp-labeled *Salmonella* cells detected by the confocal microscope than seen in the control (b) and initial biofilm (a) samples.

The bacteriophage treatment of *Salmonella* embedded in a double species biofilm was not as effective when compared to the bacteriophage treatment of the single species biofilm (Fig. 3.4); however, the bacteriophage still significantly reduced the level of *S.* Enteritidis H4717-gfp ($P<0.05$) within the biofilm on all surfaces and at all temperatures. *S.* Enteritidis H4717-gfp was

reduced by ca. 0.5, 1.5-2.0, and 0.5 log CFU/cm^2 at 20, 30, and 40°C, respectively, for all templates (Table 3.3). As observed with other treatments, the bacteriophage cocktail was most effective at 30°C.

Treatment of the attached cells with the chemical sanitizers TSP and bleach solution resulted in *Salmonella* counts below the detection limit (1 CFU/cm^2) within 1 and 3 h, respectively. In the presence of an organic layer, the attached *Salmonella* populations were reduced below the detection limit within 1 h for TSP. For bleach solution treatment, there was an initial decrease in *Salmonella* counts through 2 h followed by a period of growth through the remaining 4 h of treatment. Treatment of *Salmonella* in single and double species biofilm resulted in counts below the detection limit within 3 h for TSP and within 6 h for bleach solution (data not shown).

Discussion

The presence of *Salmonella* spp. in finished by-products can be a result of *Salmonella* surviving the heat processing or post-processing contamination. It is documented that the raw ingredients used to produce rendered by-products are contaminated with *Salmonella* and therefore serve as a potential source of cross contamination in the rendering facility (Troutt et al., 2001; Loken et al. 1968).

If contaminated, the environmental surfaces (steel, concrete, rubber, and HDPE plastic) can also contaminate the final products upon contact. Kusumaningrum et al. (2003) reported that *Salmonella* is capable of adhering to stainless steel, and that the presence of food residues increased the survival time of the bacteria. For example, when a low inoculum of *S.* Enteritidis was spread on the stainless steel with no food particles there was a 2.2 log reduction within an hour as compared with a 1.0 log reduction in the presence of milk residue. Additionally,

Kusumaningrum et al. (2003) demonstrated that the *S.* Enteritidis could be transferred from the steel to cucumber slices and chicken filets immediately or 15 minutes after contamination. Identical *S.* Typhimurium isolates were detected on a rubber conveyor belt used to transport manure 20 months after initial isolation (Sanvang et al., 2000), suggesting that the strain persisted on the conveyor belt over this extended period. Loken et al. (1968) detected *Salmonella* in 19% of 1901 swabs collected from the environmental surface of a rendering facility, which further demonstrates the potential for cross contamination between the environment and finished products.

Traditionally the environments in food processing facilities have been treated with chemical sanitizers. These sanitizers have been effective but have some disadvantages such as high cost, corrosiveness to machinery, inactivated by organic material, inactivated by variations in heat and pH, and posing a potential hazard to the environment and the workers (Marriott, 1994). This warrants the development of a new means of sanitation in the food processing industry such as bacteriophage treatment.

The use of bacteriophages offers multiple advantages: the host specificity is not likely to select for bacteriophage resistant bacterial strains, resistance against bacteriophage does not affect the efficacy of antibiotics given to humans, and bacteriophage mixtures can be easily modified and rapidly developed against resistant bacteria (Kutter and Sulakvelidze, 2005). In terms of *Salmonella* treatment, host specificity can be a hurdle for reducing contamination levels. There is not a single bacteriophage capable of lysing all known serovars of *Salmonella* (Joerger, 2003). In fact, a particular bacteriophage may not even be capable of lysing all members of a particular serovar. To overcome this shortcoming, a “cocktail” of bacteriophages must be designed capable of lysing all *Salmonella* strains of interest (Joerger 2003). The production of an

effective bacteriophage cocktail for *Salmonella* has been previously reported. Chighladze et al. reported to have developed a bacteriophage cocktail capable of lysing 232 of 245 *Salmonella* isolates composed of 21 serovars and 78 pulse field gel electrophoresis (PFGE) types (Joerger 2003).

Bacteriophage treatment for pathogen inactivation has been considered a very effective biological control method. While the majority of research involving *Salmonella* treatment with bacteriophage has focused on live animals and raw meats, it has been demonstrated that bacteriophage treatment can be successful at controlling contamination on environmental surfaces. Roy et al. (1993) applied a mixture of three bacteriophages (H387, H387-A, and 2671) to treat *L. monocytogenes* on stainless steel resulting in a 3 log reduction of *L. monocytogenes* populations. There have been limited reports on the treatment of *Salmonella* on environmental surfaces by bacteriophages; however, Chigaldze et al. were able to reduce *Salmonella* to below the detectable limit within 48 h of bacteriophage treatment on inanimate surfaces (Joerger, 2003). In the current study, the bacteriophage cocktail was able to reduce *Salmonella* by as much as 2.5 log at 30°C. MOI optimization suggested that in order for bacteriophages to propagate and lyse the host, the bacterial cells should be actively growing. This could be explained by the reliance of bacteriophages on host cell machinery for replication (Kutter and Sulakvelidze, 2005). This could also explain the variation in the efficacy of the bacteriophage treatment based on the environmental temperature.

MOI optimization during this study revealed that the bacteriophage cocktail used was capable of reducing *Salmonella* levels at a low ratio (MOI=10), but the lytic effects of the cocktail diminished after ca. 12 h of treatment (Fig. 3.2a). McLaughlin et al. (2007) reported that a single bacteriophage was incapable of eliminating *Salmonella* due to selection of resistant

strains. The presence of a resistant strain within the *Salmonella* spp. offers a possible explanation for the decreased lytic activity. While a short lytic period is not desirable, in this study, the addition of a second cocktail composed of 5 different bacteriophages resulted in suppression of *Salmonella* growth for an additional 12 h (Fig. 3.2b).

In the natural environment, bacteria may be attached to environmental surfaces as components of a large microbial network known as a biofilm. A biofilm can be composed of one or various species of bacteria and their excreted products. *Salmonella* spp. are capable of producing biofilm on surfaces such as plastic, steel, and cement (Jones and Bradshaw, 1996; Joseph et al., 2002), which are commonly found in a rendering facility. Biofilms are potential continuous sources for cross-contamination between contact surfaces and the finished rendered by-products.

In order for a bacteriophage to lyse bacteria within a biofilm the target bacteria have to be susceptible to the bacteriophage being used. Also, the exopolysaccharide (EPS) produced by the biofilm must be susceptible to degredation by the polysaccharide depolymerase produced by the bacteriophage. The effects of bacteriophage treatment on single species biofilms have been well documented. Using microscopy, Doolittle et al. (1995) discovered that the matrix produced by an *Escherichia coli* biofilm was ineffective in preventing bacteriophage T4 from infecting the bacterial cells. Karunasagar (2007) demonstrated that when using a bacteriophage concentration of 10^5 PFU/ml, *Vibrio harveyi* counts were reduced nearly 1 log CFU/cm^2 after 18 h on high density polyethylene (HDPE) plastic. Bacteriophage KH1 was able to significantly reduce ($P<0.05$) *E. coli* O157:H7 counts in biofilms from an initial concentration of 4.0 log CFU per coupon to 2.8 log CFU per coupon and 2.7 log CFU per coupon after 24 and 48 h of bacteriophage treatment, respectively (Sharma et al., 2005). The results from our study revealed

that *Salmonella* levels can also be reduced in a single species biofilm through bacteriophage treatment. Under optimal conditions (30°C), the bacteriophage cocktail was capable of reducing *S.* Enteritidis within the biofilm by ca. 3 log CFU/cm^2. While the level of reduction observed in this study is in agreement with other studies, the effect observed during the current study occurred within 6 h as opposed to the 18-48 h range reported by previous studies.

Biofilms composed of multiple bacterial species may contain additional barriers such as bacterial cell co-aggregation (Rickard et al., 2003; Sutherland 2004). Storey and Ashbolt (2001) attempted to treat biofilm composed of *Bacteroides fragilis* and *E. coli* in an urban water distribution system with bacteriophages. The bacteriophages were initially taken in by the biofilm on stainless steel and polythene surfaces; however, following the initial treatment, bacteriophage populations began to decrease rapidly, suggesting that the bacteriophages were entering the biofilm matrix but not infecting the host cells. Our current study also demonstrates that the presence of multiple species within a biofilm may create additional barriers for the bacteriophage treatment, as the addition of *P. aeruginosa* to the biofilm decreased the amount of *Salmonella* reduction as compared with single-species biofilm.

In this study the effectiveness of the bacteriophage cocktail was also compared to that of commonly used chemical sanitizers in order to determine if the bacteriohage cocktail would make an adequate replacement. Bleach solution (200 ppm sodium hypochlorite) and TSP were selected based on a survey of participating rendering facilities (Robertson, 2008). Our results indicated that both the bleach solution and TSP were more effective at reducing *Salmonella* contamination than the bacteriophage treatment. *Salmonella* inactivation by both sanitizers was achieved within 1 h for both attachment studies and within 3 h for both the single and double species biofilm. The one exception was that the bleach solution was ineffective at treating the

attached *Salmonella* cells in the presence of organic material, which can be explained by the fact that chlorine based compounds have a high affinity to bind to organic material. Once bound to organic material the compounds are inactivated (Guthrie, 1992). As compared with chemical sainitizers, bacteriophages are not corrosive to quipment and are not harmful to the environment like many chemical sanitizers do (Kutter and Sulakvelidze, 2005). The bacteriophages are cost efficient since they are self replicating and only require actively growing host cells. Furthermore, the rendering plant also has a continuous source for new bacteriophages in the incoming raw material, as a bacteriophage capable of lysing all *Salmonella* serotypes of interest was easily isolated from the raw material as demonstrated by this study.

This study has demonstrated that a bacteriophage cocktail can be used to reduce *Salmonella* on the surfaces found within a rendering facility at various temperatures and in various forms of attachment. Up to 3 log CFU/cm^2 of *Salmonella* can be inactivated by the bacteriophage cocktail within 6 h of treatment when the temperature was maintained between 20 and 40°C. By reducing or eliminating the amount of *Salmonella* contamination found within the rendering environment, the bacteriophage treatment may decrease the potential for cross-contamination leading to a microbiologically safe animal meal. The treatment can be used without any concern for potential hazards to the finished animal meal, rendering equipment, humans, or the animals consuming the finished feed. While more work is needed with multi-species biofilm, the data presented in this study suggests that bacteriophage treatment can be used by the rendering industry to lower contamination levels of not only *Salmonella* but any pathogens of concern.

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Figure Legend

Fig. 3.1. Transmission electron microscopic images of individual bacteriophages. J161 (a), M226 (b), H3353S6p2(c), and H4717S5p2 (d) observed at 100,000X.

Fig. 3.2. MOI optimization of the bacteriophage cocktail with one application of cocktail (a) and two cocktails (b). Control (×), MOI 1 (▲), MOI 10 (■), and MOI 100 (♦). "↓" on (b) signals the addition of the 2nd cocktail.

Fig. 3.3. Bacteriophage treatment of *Salmonella* attached to concrete (a), plastic (b), rubber (c), and steel (d) at 20° (♦), 30° (▲), and 40°C (●). Control and treatment were plotted in solid and dashed lines, respectively.

Fig. 3.4. Confocal images of single and double species biofilm on stainless steel at 0h (a, d), 6h of control (b, e), and 6h of bacteriophage treatment (c, f).

Table 3.1. Bacteriophages isolated from chicken offal

Offal Sample	Treatments	Serotypes Affected	Bacteriophage ID
A	A1	5*, 6, 7, 12, 16	IA1, JA1, LA1, OA1, TA1
	A2	7, 12, 16	LA2, OA2, TA2
B	B1	1, 6, 7, 10, 11, 12, 16	AB1, JB1, LB1, MolB1, MonB1, OB1, TB1
	B2	1, 3, 6, 7, 10, 11, 12, 16	AB2, DB2, JB2, LB2, MolB2, MonB2,
C	C1	2, 6, 7, 12, 13, 16	BC1, **JC1****, LC1, OC1, PC1, TC1
	C2	2, 3, 7, 12, 13, 16	BC2, DC2, LC2, OC2, PC2, TC2
D	D1	1, 3, 5, 6, 7, 9, 12, 13, 16	AD1, DD1, ID1, JD1, LD1, MbD1, OD1, PD1, TD1
	D2	1, 3, 7, 9, 12, 13, 16	AD2, DD2, LD2, MbD2, OD2, PD2, TD2
E	E1	1, 2, 3, 6, 7, 9, 12, 13 ,15, 16	AE1, BE1, DE1, JE1, LE1, MbE1, OE1, PE1, SE1, TE1
	E2	1, 2, 3, 5, 6, 7, 9, 12, 13, 15, 16	AE2, BE2, DE2, IE2, JE2, LE2, **MbE2**, PE2, SE2, TE2
F	F1	2, 3, 6, 7, 12, 16	BF1, DF1, JF1, OF1, TF1
	F2	2, 3, 6, 7, 12, 16	BF2, DF2, JF2, LF2, OF2, TF2

**Salmonella serotpye: 1. S. Amsterdam, 2. S. Banana, 3. S. Demerara, 4. S. Enteritidis, 5. S. Idikan, 6. S. Johannesburg, 7. S. Livingstone, 8-9. S. Mbandaka, 10. S. Molade, 11. S. Montevideo, 12. S. Oranienburg, 13. S. Putten, 14-15. S. Senftenberg 16. S. Typhimurium*

***Bold print indicates the bacteriophage was used during treatment studies*

Table 3.2. Effect of residual bacteriophage on *Salmonella* enumeration

Time (h)	Non-centrifuged	Centrifuged
1	2.93*±0.07^{A**}	3.09±0.07^{A}
2	2.76±0.06^{A}	2.85±0.08^{A}
3	2.69±0.09^{A}	2.71±0.06^{A}
4	2.41±0.12^{A}	2.52±0.08^{A}
5	2.28±0.05^{A}	2.37±0.05^{A}
6	2.19±0.09^{A}	2.24±0.05^{A}

**log CFU/cm2*

*** The letters represent the levels of significant difference (P<0.05) between the two test groups*

Table 3.3. *Salmonella* levels following 6 h of bacteriophage treatment (MOI 10) on various surface templates as affected by temperature

Contamination	Surface	20°C		30°C		40°C	
		Control	Treatment	Control	Treatment	Control	Treatment
Attachment in buffer	Concrete	3.67*±0.15^{A**}	2.50±0.22^{B}	3.97±0.16^{A}	1.97±0.18^{B}	3.92±0.13^{A}	2.35±0.18^{B}
	Plastic	3.68±0.22^{A}	2.34±0.13^{B}	4.19±0.09^{A}	2.11±0.13^{B}	3.84±0.19^{A}	2.06±0.13^{B}
	Rubber	3.47±0.20^{A}	2.29±0.06^{B}	4.04±0.14^{A}	1.56±0.20^{B}	3.90±0.10^{A}	2.03±0.15^{B}
	Steel	3.55±0.09^{A}	2.25±0.13^{B}	4.22±0.11^{A}	2.22±0.20^{B}	3.92±0.19^{A}	2.18±0.14^{B}
Attachment in organic layer	Concrete	3.84±0.09^{A}	2.65±0.19^{B}	4.06±0.10^{A}	1.79±0.15^{B}	4.17±0.12^{A}	2.95±0.14^{B}
	Plastic	3.56±0.17^{A}	2.34±0.12^{B}	3.82±0.23^{A}	1.87±0.14^{B}	3.88±0.10^{A}	2.41±0.19^{B}
	Rubber	3.62±0.17^{A}	2.59±0.21^{B}	4.43±0.22^{A}	1.78±0.20^{B}	3.96±0.23^{A}	2.16±0.12^{B}
	Steel	4.03±0.10^{A}	2.62±0.16^{B}	4.28±0.08^{A}	2.21±0.12^{B}	4.03±0.10^{A}	2.39±0.10^{B}
Single Species Biofilm	Concrete	5.03±0.10^{A}	3.59±0.12^{B}	5.46±0.17^{A}	2.47±0.10^{B}	4.48±0.12^{A}	3.30±0.19^{B}
	Plastic	5.02±0.09^{A}	3.24±0.15^{B}	5.79±0.10^{A}	2.06±0.15^{B}	4.78±0.09^{A}	3.19±0.08^{B}
	Rubber	5.05±0.09^{A}	3.43±0.14^{B}	5.52±0.12^{A}	3.73±0.16^{B}	4.47±0.10^{A}	3.20±0.14^{B}
	Steel	4.97±0.14^{A}	3.01±0.08^{B}	5.63±0.19^{A}	2.82±0.19^{B}	4.57±0.11^{A}	3.36±0.17^{B}
Double Species Biofilm	Concrete	4.85±0.18^{A}	4.50±0.14^{B}	5.24±0.12^{A}	3.74±0.15^{B}	4.84±0.16^{A}	4.41±0.19^{B}
	Plastic	4.56±0.09^{A}	4.20±0.09^{B}	4.89±0.16^{A}	3.05±0.10^{B}	4.53±0.10^{A}	4.17±0.12^{B}

Rubber	4.47 ± 0.11^{A}	4.07 ± 0.12^{B}	4.97 ± 0.14^{A}	3.44 ± 0.15^{B}	4.59 ± 0.16^{A}	4.18 ± 0.08^{B}
Steel	4.78 ± 0.22^{A}	4.49 ± 0.12^{B}	4.84 ± 0.13^{A}	3.04 ± 0.09^{B}	4.43 ± 0.10^{A}	4.04 ± 0.13^{B}

**Salmonella counts CFU/cm^2*

***The different letters represent significant differences (P<0.05) between the control group and treated group after 6 h incubation*

Figure 3.1

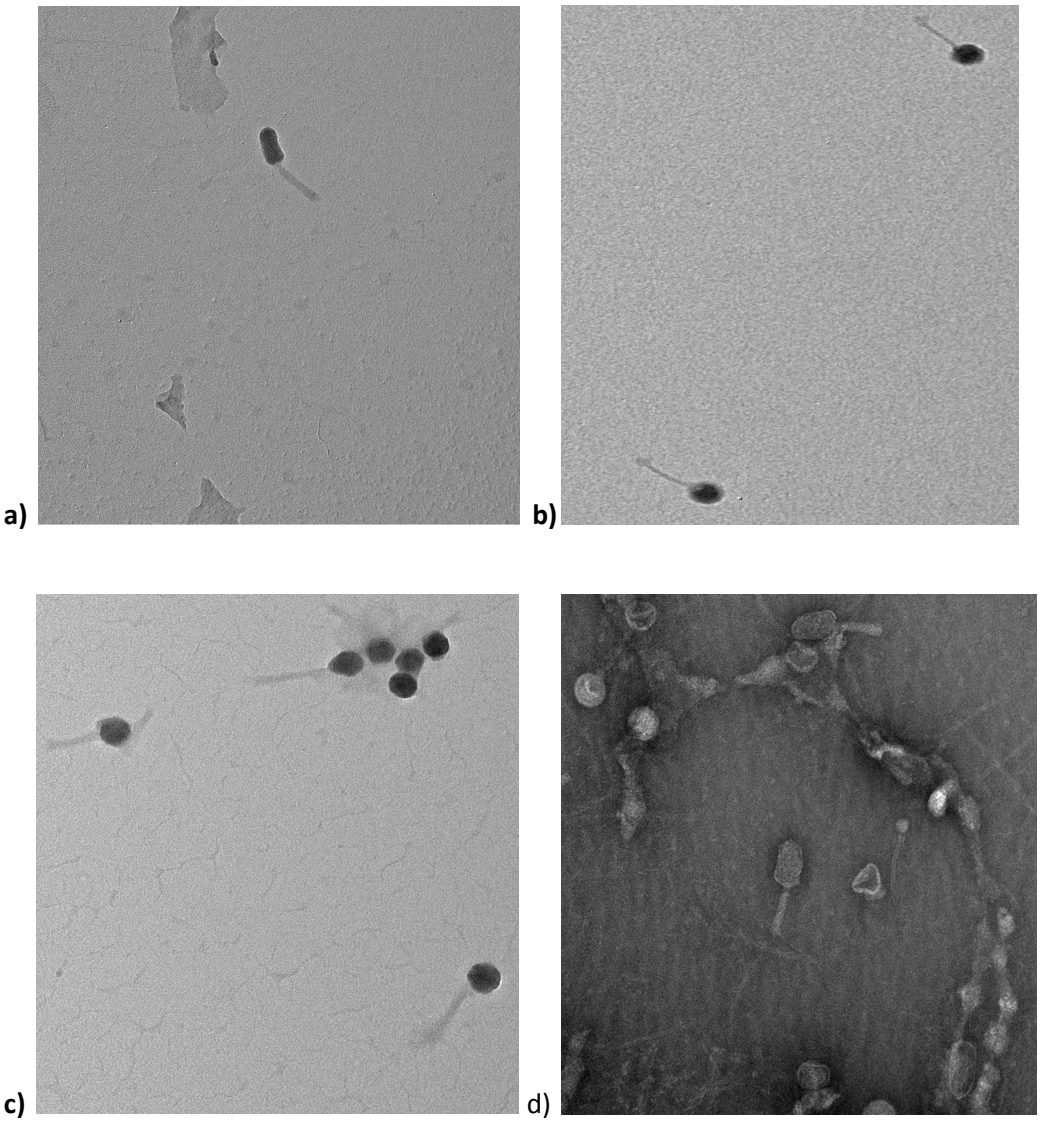
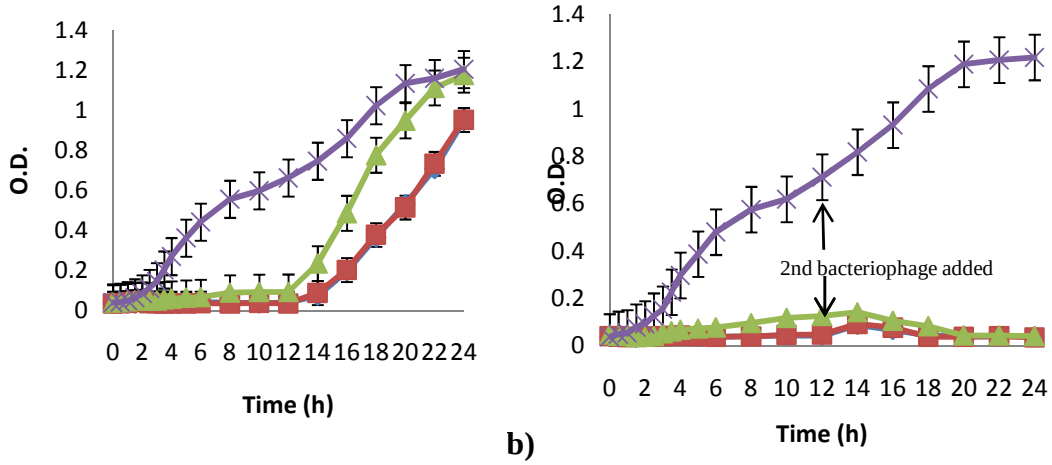
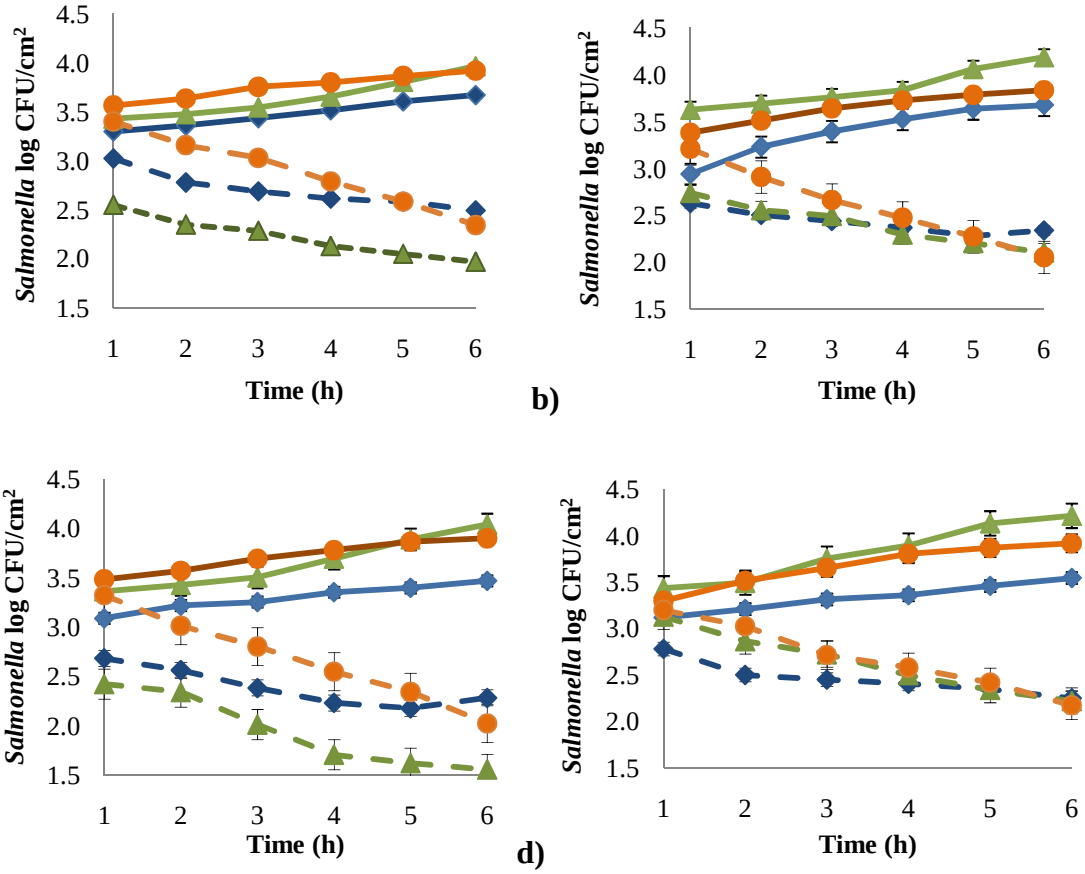


Figure 3.2



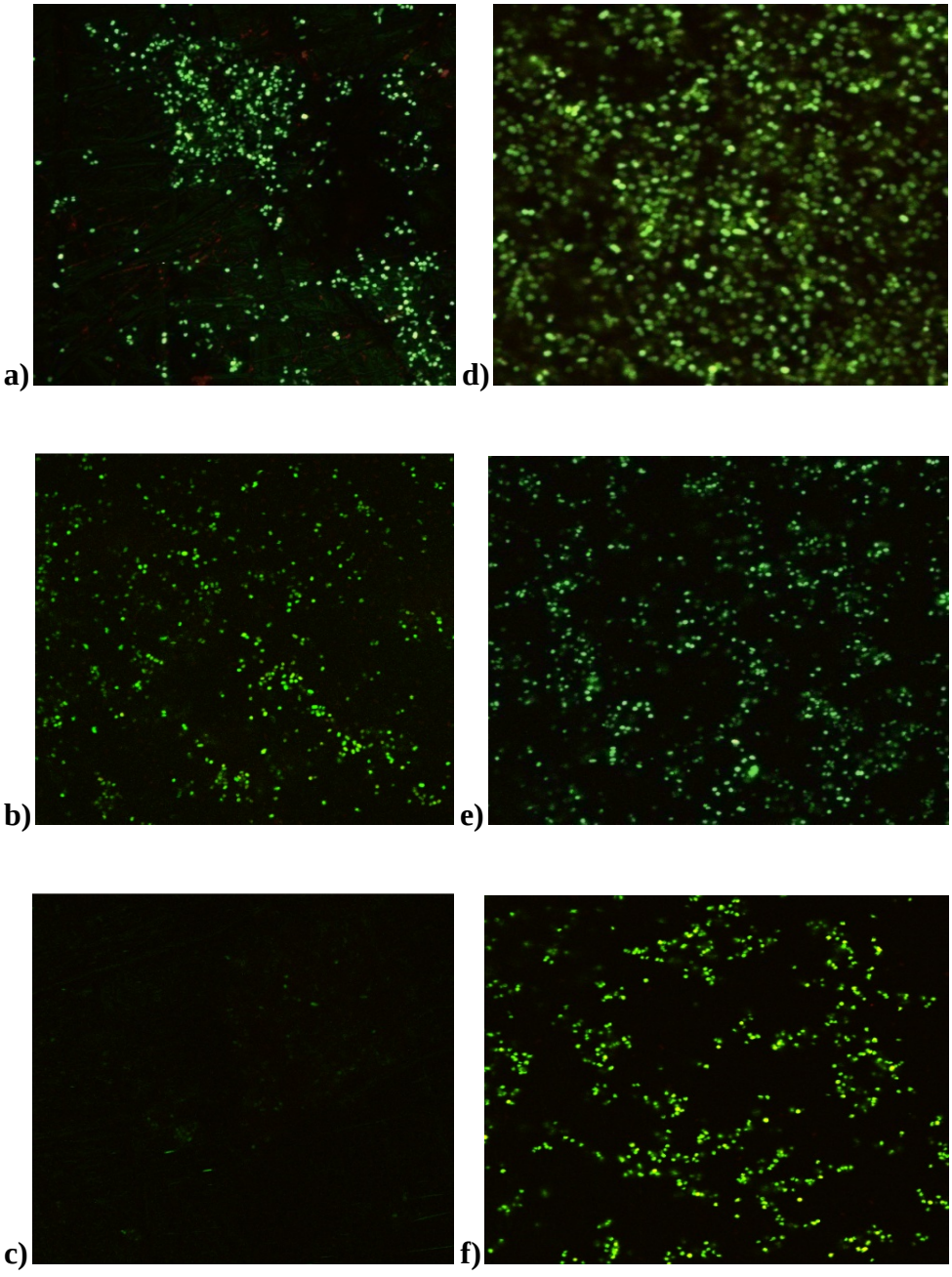
a) b)

Figure 3.3



a) b) c) d)

Figure 3.4



CHAPTER 4

BACTERIOPHAGE TREATMENT OF RAW CHICKEN OFFAL, RENDERED ANIMAL MEALS, AND MICE CHALLENGED WITH *SALMONELLA* ENTERITIDIS

Abstract

The objectives of this study were to determine if a bacteriophage cocktail could be used either to reduce *Salmonella* in the raw material used by the rendering industry or as a feed additive to reduce *Salmonella* infection in animals. A cocktail of 5 previously isolated bacteriophages with a multiplicity of infection (MOI) of 10 and 100 was used to treat sterile and non-sterile raw material contaminated with a *Salmonella* cocktail (ca. 10^5 CFU/g) at 20°, 30°, and 40°C. The bacteriophage treatment reduced *Salmonella* levels by 2.0, 2.7, and 2.5 log CFU/g at 20°, 30°, and 40°C in the sterile poultry offal samples, as compared with a 2.0, 2.2, and 2.2 log CFU/g reduction, respectively, in the non-sterile samples. Bacteriophage stability testing indicated that the titer of lyophilized bacteriophages mixed in animal meals decreased by 1.5 log PFU/g at 30°C, while fresh bacteriophage levels decreased by ca. 0.23 log PFU/g at 4°C over 4 weeks. The lyophilized bacteriophage did not reduce *Salmonella* levels in the animal meals during storage at 30°C, but *Salmonella* growth was slowed down during the rehydration step. During the 4-week trial, the difference in *Salmonella* levels after rehydration for 24 h between those samples treated with lyophilized bacteriophage and those not treated ranged from 0.8-1.5 and 1.0-2.0 log CFU/g when using a MOI of 10 and 100, respectively. The addition of fresh bacteriophages to the rehydrated meals was able to reduce *Salmonella* levels by ca. 1.0 log CFU/g at 30°C. The bacteriophage was also tested for reducing *Salmonella* infection of Balb/c mice. Mice receiving feed supplemented with the bacteriophage cocktail (MOI of 100) showed no signs of infections (shedding in feces or histological analysis), while mice not receiving the

bacteriophages shed *Salmonella* in the feces for a period of 2.5 weeks and histological examination revealed signs of inflammation in the liver. These results indicate that the bacteriophage cocktail can be used to significantly reduce *Salmonella* levels in raw chicken offal, which reduces the probability of cross-contamination, and can be used as an effective feed additive if added in liquid form shortly before consumption by animals.

Introduction

In the U.S., approximately, 100 million hogs, 35 million cattle, and 8 billion chickens are slaughtered for consumption annually, resulting in a large quantity of inedible by-products (Meeker and Hamilton, 2006). On average, approximately 50% of an animal is inedible (Ockerman and Hansen, 1988; Meeker and Hamilton, 2006). Of the potential treatment methods (pyre, incineration, landfill, burial, and rendering), the rendering process is the only method that adequately eliminates the biological risks without hazardous environmental impact (Meeker and Hamilton et al., 2006; U.K. Department of Health, 2001).

The raw material coming into a rendering facility can be highly contaminated with numerous human pathogens. Troutt et al. (2001) detected *Clostridium perfringens*, *Listeria* spp., *Campylobacter* spp., and *Salmonella* spp. in 71.4, 76.2, 29.8, and 84.5% of raw poultry offal samples, respectively. An U.S. federal study conducted between January 1998 and December 2000 reported the following *Salmonella* contamination rates: 20% in broilers, 8.7% swine, and 2.7% for cows and bulls (Rose et al., 2002). The Food Safety Inspection Service (FSIS) of the USDA reported *Salmonella* in ca. 16% of broiler carcasses sampled in 2005 (D'Aoust and Maurer, 2007). *Salmonella* contamination levels ranged from 6.5-7.2 log CFU/g from poultry feathers and 5.8-6.3 log CFU/g on poultry carcasses (Kotula and Pandya, 1995). Botteldoorn et al. (2003) reported an average *Salmonella* contamination rate of 37% on swine carcasses at 5

different slaughter houses. The rates of *Salmonella* contamination were as high as 70% dependent upon the slaughter house and date of sampling.

While the raw material may contain *Salmonella*, the rendering system is adequately designed to inactivate pathogen contamination from the raw material. The cookers used to treat the incoming material are heated by boiler steam so that the internal temperature of the raw material reaches 115 to 145°C (Anderson, 2006). However, Hacking et al. (1992) tested finished meat and feather meal from Ontario rendering facilities over a four month period and found *Salmonella* contamination rates of 81% and 40%, respectively. In the U.S., a FDA study conducted by McChesney et al. (1995) detected *Salmonella enterica* in 56% of the 101 meal samples tested. Our previous study demonstrated that *Salmonella* isolates collected from finished rendered products were heat sensitive with an average D-value of 45 sec at 65° C (Kinley at al., 2009), suggesting the finished products may have been contaminated with *Salmonella* post-processing. This study also revealed that a persistent strain of *Salmonella* was not found in the finished products of a given rendering facility using pulse field gel electrophoresis (PFGE) analysis (Kinley et al., 2009). This suggests that the cross-contamination is resulting from a new incoming source such as the raw material.

Regardless of the source, the finished product can serve as a reservoir for *Salmonella* contamination which will then be incorporated into animal feed and into the food chain. Epidemiological studies have linked human *Salmonella* spp. to contaminated animal feed (Crump et al. 2002). For example, a *S.* Heidelberg outbreak in 1963 led to 77 infections from milk consumption, which was linked to bovine mastitis caused by the same *Salmonella* serotype, which was found in the meat and bone meal fed to the cows (Knox et al. 1963).

Currently, bacterial feed contamination is treated through organic feed additives such as Sal Curb®. Larsen et al. (1993) demonstrated that the addition of Sal Curb® to poultry feed reduced *Salmonella* by ca. 2 log CFU/g. Opitz et al. (1993) further demonstrated that when added into poultry feed Sal Curb® can significantly ($P<0.05$) reduce the rate of *S.* Enteritidis infection in chicks; however, the levels of *S.* Enteritidis in the feces of infected chicks (1.18 log CFU/g) were not significantly less than the control. In addition to Sal Curb®, Opitz et al. (1993) tested the potential of probiotics [Primalac®, egg powder, lactose, and *Lactobacillus acidophilus* (Lactbio®)] as anti-*Salmonella* feed additives. They found that none of the tested additives significantly ($P<0.05$) reduced the rate of *S.* Enteritidis infection and reduced levels in those animals infected, which led them to conclude none of the tested additives should be solely relied upon for prevention of *S.* Enteritidis infection.

An alternative to these chemical-based additives is bacteriophages. Bacteriophages, viruses with specific bacterial hosts, have been used as a biological agent for pathogen control. Recent studies have indicated that bacteriophages can be used as a therapeutic agent for *Salmonella*. Berchieri et al. (1991) applied bacteriophages to reduce the level of *Salmonella* by 1 log CFU/g within 12 h in the crop, intestine and cecae of chickens.

The objectives of this study were to determine if treatment with a bacteriophage can reduce *Salmonella* levels within the raw material used by the rendering industry and if a bacteriophage cocktail can potentially be used as a feed additive to reduce *Salmonella* population upon rehydration of the feed *in vitro* and *in vivo*.

Materials and Methods

Bacteriophage cocktail preparation: The bacteriophage cocktail used during this study was composed of five bacteriophages previously isolated and purified from raw chicken offal

(n=2), inactivated sludge (n=2), as well as the F0 bacteriophage from the University of Calgary's *Salmonella* Genetic Stock Centre (Kinley et al., unpublished). The bacteriophage stocks were prepared and stored in sterile SM buffer (NaCl 5.8 g l^{-1}, $MgSO_4 \cdot 7H_20$ 2 g l^{-1} & Tris-HCl 1 M, pH 2.5, 50 ml l^{-1}).

Sample preparation: Incoming raw chicken offal was collected from a SC rendering facility. Upon arrival at the lab, the raw material was mixed with sterile saline (1:1), and blended in a sterile laboratory blender (Waring model 51BL30) at Hi speed for 5 min. To enumerate *Salmonella*, serial dilutions of sample suspensions were made using sterile saline and spread plated onto xylose lysine tergitol-4 (XLT-4, Becton-Dickinson and company). The blended raw material, placed inside 50 ml sterile centrifuge tubes, was then frozen at -80°C for subsequent bacteriophage treatment.

Bacteriophage treatment of raw materials: Half of the prepared raw samples were sent to the physics department at Auburn University via overnight delivery, where they were irradiated at 1.5 MRad at 6,133 R/min. Both sterile and non-sterile raw material was divided into 8 ml portions in sterile whirl-pak bags in preparation for bacteriophage treatment. Overnight cultures of rifampin resistant (100 µg/ml) *Salmonella* isolates (Typhimurium, Enteritidis, Mbandaka, Johannesburg, and Idikan) were added together as a cocktail and then mixed into the sterile and non-sterile raw material at a final concentration of 10^5 CFU/ml. The bacteriophage cocktail was then added to each experimental bag at a final concentration of 10^6 PFU/ml or 10^7 PFU/ml. The same amount of SM buffer was added to the control group in place of the bacteriophage cocktail. All samples were then incubated at 20, 30, and 40°C for 6 h. Every hour, raw material suspensions were removed and centrifuged (5,000 rpm) for 10 min and the supernatant was poured off to separate bacteriophages from the sample. The pellets were then

washed once in sterile saline and then suspended in sterile saline. Serial dilutions were spiral-plated with an Autoplate 4000 (Spiral Biotech Norwood, MA) onto XLT-4/rif (100 µg/ml) for *Salmonella* enumeration at 37° C for 24 h.

Bacteriophage treatment of *Salmonella* in finished rendered animal meals upon rehydration: The bacteriophage cocktail and *Salmonella* were suspended in sterile 10% skim milk and tryptic soy broth (TSB), respectively, and lyophilized using a Vir Tis Model 7.0 freeze dryer (SP industries NY) for ca. 24 h. Irradiated (1.5 MRad at 6,133 R/min) blood, feather, and poultry meals were divided into four treatment groups each. Group 1 had lyophilized *Salmonella* cocktail added at a concentration of 10^5 CFU/g. Group 2 had both lyophilized bacteriophage and *Salmonella* cocktails added at concentrations of 10^6 PFU/g or 10^7 PFU/g and 10^5 CFU/g, respectively. Group 3 had lyophilized bacteriophage cocktail added at a concentration of 10^6 PFU/g or 10^7 PFU/g. Group 4 had no additives. The samples were kept in sterile whirl-pak bags at 30°C for a period of 1 month.

For the rehydration study, samples from groups 1 and 2 were collected weekly and suspended into buffered peptone water. Samples from group 3 and 4 were collected weekly and suspended into buffered peptone water that was inoculated with the *Salmonella* cocktail at a concentration of 10^5 CFU/ml. The samples were incubated at 30°C for 24 h with samples taken at pre-determined time intervals. The samples were centrifuged and the supernatant was poured off to remove bacteriophages from the sample. The pellets were washed as described for the raw meat samples and then suspended in sterile saline. Serial dilutions were made with sterile saline and spiral plated onto XLT-4. All plates were incubated at 37°C and enumerated after 24 h.

An additional trial was conducted as described above with lyophilized bacteriophage at a concentration of 10^6 PFU/g. During this additional study, a second bacteriophage cocktail

(suspended in SM buffer) was added to the experimental groups at a MOI of 10 at the 12h sampling time.

Stability of lyophilized bacteriophages in dry meals: In addition to testing the lytic effectiveness of the lyophilized bacteriophage, the stability of the bacteriophage cocktail in dry meals was determined as well. The supernatant removed during *Salmonella* enumeration was added to molten TSA along with the *Salmonella* cocktail (10^5 CFU/ml). The molten TSA was overlayed onto TSA and incubated for 24 h at 37°C. Following incubation, plaque counts were made to determine the amount of bacteriophage reduction over the course of the month-long trial.

The stability of the liquid phage in the dry rodent feed was also determined at refrigeration temperatures (4°C). The bacteriophage cocktail (1 ml) was added to dry, sterile rodent feed at a concentration of 10^8 PFU/g. The feed was kept inside sterile-whirl pak bags at 4°C for 4 weeks. Samples were removed weekly and suspended in sterile saline. The bacteriophage titer was determined as described above using the agar overlay method.

***In vivo* study with Balb/c mice:** Female Balb/c mice (4-6 weeks, Harlan Laboratories) were housed in sterile micro-isolater cages with sterile bedding at the Godley Snell Research Center (Clemson, SC). The mice (n=10) were divided into two groups equally. All mice were given water containing gentamicin (4 mg/l) to reduce background microbial population. The experimental group were given a dough diet (Bio-Serv) supplemented with the bacteriophage cocktail at a MOI of 100. The control mice were given the dough diet with no additives. After 7 days of acclimation, feed was removed for 24 h. Following this 24 h period, all mice were anesthetized with 2.5% isofluorane mixed with 1.5% oxygen and orally administered 0.20 ml of *S.* Enteritidis H4717 (10^6 CFU/mouse) using gavage needles. After oral challenge, the mice

were returned to their respective diets for a period of 4 weeks. Both the weight and behavior of each mouse was observed for the duration of the experiment. All procedures were approved by Clemson University's Institutional Animal Care and Use committee.

Analysis of *Salmonella* levels in fecal content during mouse trial: Twenty-four hours after arrival of the mice, one gram of feces from each cage was collected aseptically and homogenized in sterile saline using a stomacher 400 circulator (Seward) for 5 min. Samples were enriched in Rappaport-Vassiliadis (RV) broth for 24 h followed by spread-plating onto XLT-4 to ensure the mice were free of *Salmonella*.

Feces samples were collected and homogenized from each cage at 24 h post-infection as described above. Homogenized samples were centrifuged at 5,000x rpm and the supernatant removed. The pellet was washed and resuspended in sterile saline. Serial dilutions were made in sterile saline and spiral-plated onto XLT-4/ rif. *Salmonella* levels were determined following 24 h incubation at 37°C. Fecal sampling was conducted in the same manner twice weekly during the 4 week trial.

Analysis of *Salmonella* levels and histological analysis of intestinal and liver tissue collected during the mouse trial: At the end of the 4-week trial, all mice were euthanized using isofluorane. The liver and intestine were aseptically removed from 2 mice in each group. Approximately 50% of each organ was collected and suspended in sterile saline for *Salmonella* enumeration, and the remaining tissues were sent to the Clemson Veterinary Diagnostic Center (Columbia, SC) for histological analysis.

For enumeration, each tissue sample was weighed out, and one gram samples were homogenized in sterile saline using a glass tissue grinder. The homogenized samples were then

used to make serial dilutions in sterile saline. The dilutions were spiral-plated onto XLT-4/rif and incubated at 37°C for 24 h.

For histological analysis, the tissue samples were fixated in 10% formalin, cut, and embedded in paraffin. Cross-sections were mounted on glass slides, air dried, and stained with hematoxylin and eosin (H&E). Pathological analysis was then performed on all tissue samples by one pathologist.

Statistical analysis. All *Salmonella* counts were converted into log values. The mean counts and standard deviations were determined for the samples. The data were subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at $P = 0.05$. The Tukey's multiple comparison procedure of the Statistical Analysis System (SAS 2001, Cary, NC) was used to compare all pairs of means when the test criterion for the analysis of variance was significant.

Results

Salmonella enumeration from the raw chicken offal revealed a contamination level of ca. 10^5 CFU/g (data not shown). Treatment of the raw chicken offal was effective at both a MOI of 10 and 100, with no significant difference ($P>0.05$) in the effectiveness of the two (Fig. 4.1). After 6 h of incubation the average differences between *Salmonella* levels in the bacteriophage treated (MOI of 10 and 100) and non-treated groups were ca. 2.0, 2.7, and 2.5 log CFU/g in the sterile chicken offal at 20°, 30°, and 40°C, respectively (Fig. 4.1a, b, c). The differences between *Salmonella* levels in the bacteriophage treated (MOI of 10 and 100) and non-treated groups in non-sterile offal were ca. 2.0, 2.2, and 2.2 log CFU/g at 20°, 30°, and 40°C, respectively (Fig. 4.1d, e, f). In the control group, *Salmonella* grew ca. 0.5 log CFU/g more in the sterile offal as compared with the non-sterile offal.

Experiments were designed to study the suppressive effect of lyophilized bacteriophage on the growth of *Salmonella* during rehydration of the contaminated animal meals (blood, feather, and poultry) (Table 4.1). *Salmonella* counts in the rendered meals decreased by ca. 1 log CFU/g during the 4 week study (Fig. 4.2). During the rehydration step, the changes in *Salmonella* counts within blood meal in the presence of lyophilized bacteriophage (MOI of 10 and 100) are presented in Table 4.1a. In the blood meal, there was ca. a 1.5, 1.5, 1.0, and 0.8 log CFU/g difference between the control and treated (MOI of 10) samples at weeks 1-4, respectively, as compared with ca. a 2.0, 1.5, 1.5, and 1.0 log CFU/g difference between the control and treated samples when using a MOI of 100. The effect of the lyophilized bacteriophage on *Salmonella* in both the feather and poultry meal resulted in similar differences (Tables 4.1b-c).

To determine if the lyophilized bacteriophage can prevent the contamination of animal meals during the rehydration step, fresh *Salmonella* was added to the meals containing the bacteriophages (treatment group 3). The level of *Salmonella* reduction was similar to those observed when the *Salmonella* was already present in the meal (Table 4.1). In both studies, there was no significant difference ($P>0.05$) in *Salmonella* levels between the control and bacteriophage-treated samples in all meals after 24 h (data not shown).

To determine if a fresh, liquid culture of bacteriophage could reduce *Salmonella* levels in rehydrated animal meals, a different bacteriophage cocktail (MOI of 10) suspended in SM buffer was added directly to the meals following 12 h of rehydration (treatment group 1). The difference in *Salmonella* growth in the control and treated samples was in the range of 0.7-1.4 log CFU/g after the remaining 12 h of incubation. The fresh bacteriophage reduced the amount of *Salmonella* by ca. 1 log CFU/g in all meal types (Table 4.2).

Analysis of the stability of the lyophilized bacteriophage cocktail within the dry finished meals at 30°C, revealed that the concentration decreased by ca. 1.5 log PFU/g over the 4-week test period (Fig. 4.3). Stability testing of the fresh bacteriophage inoculated into the animal feed used for the mice study at 4°C revealed that the bacteriophage concentration had ca. a 3% decrease (0.23 log PFU/g) over the 4-week trial (Fig. 4.4).

To determine the feasibility of using bacteriophage as a feed additive for *Salmonella* control, a small animal study was conducted. Our preliminary study suggested that *Salmonella* infection in mice follows a dose dependent pattern. At inoculation levels of $5x10^8$ *Salmonella* CFU/mouse, all mice were killed by the end of a two week trial. By reducing the initial inoculation level to $5x10^6$ CFU/mouse, our animal study revealed that no *Salmonella* was detected in the fecal samples from the experimental group throughout the 4-week trial; however, *Salmonella* was detected in the fecal samples from the control group up to 2.5 weeks (Table 4.3). The liver and intestinal tract of both the control and experimental mice were free of *Salmonella* 4-weeks post-infection as determined by the plate-counting method. The histological analysis gave no indications of inflammation associated with *Salmonella* infection in the liver and intestine of the experimental mice; however, it did reveal venous dilation in the liver of the control mice. In the liver of one control sample, monocytes were found in the portal areas and cholangiohepatitis was observed. The intestine for the control mice showed no signs of inflammation.

Discussion

Salmonella is an important foodborne pathogen affecting the microbial safety of rendered animal by-products. A study conducted by Watkins et al. (1959) detected *Salmonella* in 18.5% of the finished rendered products tested; whereas in our previous study, *Salmonella* was detected in

8.7% of the finished rendered samples tested (Kinley et al., 2009). The current study demonstrated that the level of *Salmonella* present in finished product decrease at a rate of 1 log CFU/g per month, suggesting the pathogen can persist for long periods of time within these products. The presence of *Salmonella* in the finished products can result from either insufficient heat processing or post-processing contamination. The previous study by Kinley et al. (2009) suggests that contamination may arise from cross-contamination. The *Salmonella* isolates collected during that study had a maximum D-value of 45 sec at 65°C, which is at least 50°C lower than the 115-145°C temperatures used during the rendering process (Anderson, 2006).

A likely source for cross contamination is the incoming raw material, which may have already been contaminated with pathogenic bacteria such as *Salmonella*. The current study has indicated that the average *Salmonella* count from raw chicken offal is ca. 10^5 CFU/g. This level is in agreement with the 5.8-6.3 log CFU/g of *Salmonella* on raw broiler carcasses reported by Kotula and Pandya (1995). Troutt et al. (2001) reported that 84.5% of the raw products collected from U.S. rendering facilities were contaminated by *Salmonella*.

Previous studies have revealed that treatment of raw meats is successful for *Salmonella* reduction when using a bacteriophage cocktail. For example, Higgens (2005) demonstrated that a 72 phage cocktail was capable of reducing *S.* Enteritidis (SE) by as much as 60% on poultry carcasses. Goode et al. (2003) reported that using a bacteriophage cocktail at a MOI of 100 or 1000 could decrease *Salmonella* by 2 log CFU/cm^2 on chicken skin. Fiorentin et al. (2005) demonstrated that SE levels in the rinse water of artificially contaminated chicken skin (10^6 CFU/cut) could be significantly reduced ($P<0.05$) with a bacteriophage cocktail at a MOI of 1000. In this study, the maximum observed reduction in *Salmonella* levels in raw chicken offal by bacteriophage treatment was 2.7 log CFU/g at 30° and 40°C. The lack of a significant

difference between the efficacy of the bacteriophage treatment at a MOI of 10 and 100 may suggest the bacteriophage cocktail reaches maximum efficacy at a MOI of 10. There was also no notable difference between the treatment of the irradiated offal and the offal that was not sterilized, which indicates that a bacteriophage cocktail is host-specific and is less affected by background microorganisms. This study has shown that bacteriophage treatment can serve as an effective way to lower *Salmonella* contamination in the raw material, thereby reducing the probability for cross-contamination in the rendering facility.

Aside from preventing *Salmonella* contamination in the finished product, the current study aimed to determine the potential for bacteriophage to be used as a feed additive when the finished product is already contaminated with *Salmonella*. To accomplish this, we tried to determine if the bacteriophage cocktail could remain alive within the finished rendered product. The results indicated that the bacteriophage levels decrease by 1.5 log PFU/g within 4 weeks. This relatively short shelf life means that for lyophilized bacteriophage to be effective as a feed additive, the feed would either need to be used shortly after being mixed together with bacteriophage or that the bacteriophage would have to be added at a concentration far exceeding the minimal lytic concentration. For example, in this study the bacteriophage cocktail was initially added to the meals at a MOI of 10, but the bacteriophage titers had reduced quickly during storage, resulting in a MOI of 1 within two weeks.

In this study, we also determined if bacteriophages within the feed could be reactivated upon rehydration to reduce *Salmonella* levels. The results indicated that the lyophilized bacteriophages, at a MOI of 10 and 100, was unsuccessful at eliminating the growth *Salmonella* in the feed during rehydration; however, there was a significant ($P<0.05$) difference in *Salmonella* levels between samples containing bacteriophage and those without. The

bacteriophages did slow the rate of *Salmonella* growth (average 2 log reduction as compared with the control) through 12 h of rehydration. These results suggest that *Salmonella* can be controlled to some extent by bacteriophage treatment when the rendered meal becomes rehydrated upon animal consumption. While there are no known studies using lyophilized bacteriophages for pathogen control, Kim et al. (2007) added a bacteriophage cocktail to reconstituted infant formula and reported that the bacteriophages were able to reduce *Enterobacter sakazakii* (OD_{600}=0.5) levels by 1 log CFU/g after at 24°C using the bacteriophage cocktail at a MOI of 1 and 10 for 12 h. In this study, we were able to reduce *Salmonella* level by ca. 1 log CFU/g after a 12 h period in the rehydrated meals using fresh bacteriophage. The results suggest that the lyophilized bacteriophage may require a recovery period while fresh bacteriophage is already active when it is added to the rehydrated meal.

There have been many studies indicating the potential of bacteriophages as therapeutic agents. Initial bacteriophage therapeutic trials date back to the work of d'Herelle (1921). He was able to use bacteriophage treatment to reduce the occurrence of avian typhosis in chicken flocks across France. D'Herelle (1926) also demonstrated that water supplemented with bacteriophage prevented *Pasteurella multocida* infection in water buffalo. More recently, Smith and Huggins (1983) demonstrated that a bacteriophage cocktail could be used to reduce the mortality rate due to *E. coli* O9:K30.99 infection from 100 to 17% in calves. Berchieri et al. (2001) showed that a bacteriophage cocktail could be used to reduce *S.* Typhimurium levels in the intestine, crop, and cecae by 1 log within 12 h of bacteriophage administration. The study by Berchieri et al (2001) and the current study both demonstrate that bacteriophage therapy can be effective at reducing *Salmonella* if given before the pathogenic cells can enter the epithelial cells. In our animal trial, the experimental mice were given the bacteriophage supplemented feed a week prior to

inoculation. The results of fecal sampling indicated that the bacteriophages were able to prevent *Salmonella* infection immediately after pathogen challenge. Fecal shedding of *Salmonella* in the control mice indicated that *Salmonella* successfully infected the host; however, the tissue analysis indicated that at the end of the 4-week trial, *Salmonella* was no longer detected in any of the mice. Histological analysis did not detect any signs of inflammation due to *Salmonella* infection in the experimental mice; whereas, the control mice had some signs of inflammation associated with *Salmonella* infection in the liver which suggested the mice were initially infected.

This study has demonstrated that a bacteriophage cocktail can be used to effectively reduce *Salmonella* populations within the raw material used for rendering at temperatures ranging from 20-40°C, which reduces the probability of cross-contamination. The presence of background microorganisms does not significantly affect the efficacy of the bacteriophage treatment. It was also shown that the stability of the bacteriophage as a feed additive is maintained when the bacteriophage is added n liquid form and stored at refrigeration temperatures. The study also suggests that the presence of lyophilized bacteriophages in rehydrated meals does not eliminate *Salmonella* growth but slows the rate of *Salmonella* growth during the rehydration step. By adding fresh bacteriophages to the rehydrated meals, *Salmonella* populations were reduced by ca. 1 log CFU/g, suggesting that fresh bacteriophage has potential as a feed additive. This was further supported by the animal study, where mice given feed supplemented with fresh bacteriophage prior to inoculation showed no indications of *Salmonella* infection. Overall results have demonstrated that fresh bacteriophage may be effective at reducing *Salmonella* levels at the farm level when added close to the time of consumption;

however, additional animal testing is needed to optimize bacteriophage treatment across various species due to differences in the gastrointestinal systems between animals.

Acknowledgements

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Figure legends

Fig. 1. Bacteriophage treatment of *Salmonella* in sterile raw chicken offal at 20° (a), 30° (b), and 40°C (c), and non-sterile raw chicken offal at 20° (d), 30° (e), and 40°C (f). Symbols were Control (♦), MOI of 10 (■), and MOI of 100 (▲).

Fig. 2. Stability of *Salmonella* in finished rendered meal over at 30°C.

Fig. 3. Stability of lyophilized bacteriophage with MOI of 10 and 100 infinished rendered meal. Symbols were MOI of 10 (■) and MOI of 100 (♦).

Fig. 4. Stability of bacteriophage in animal feed stored at 4°C.

Figure 4.1

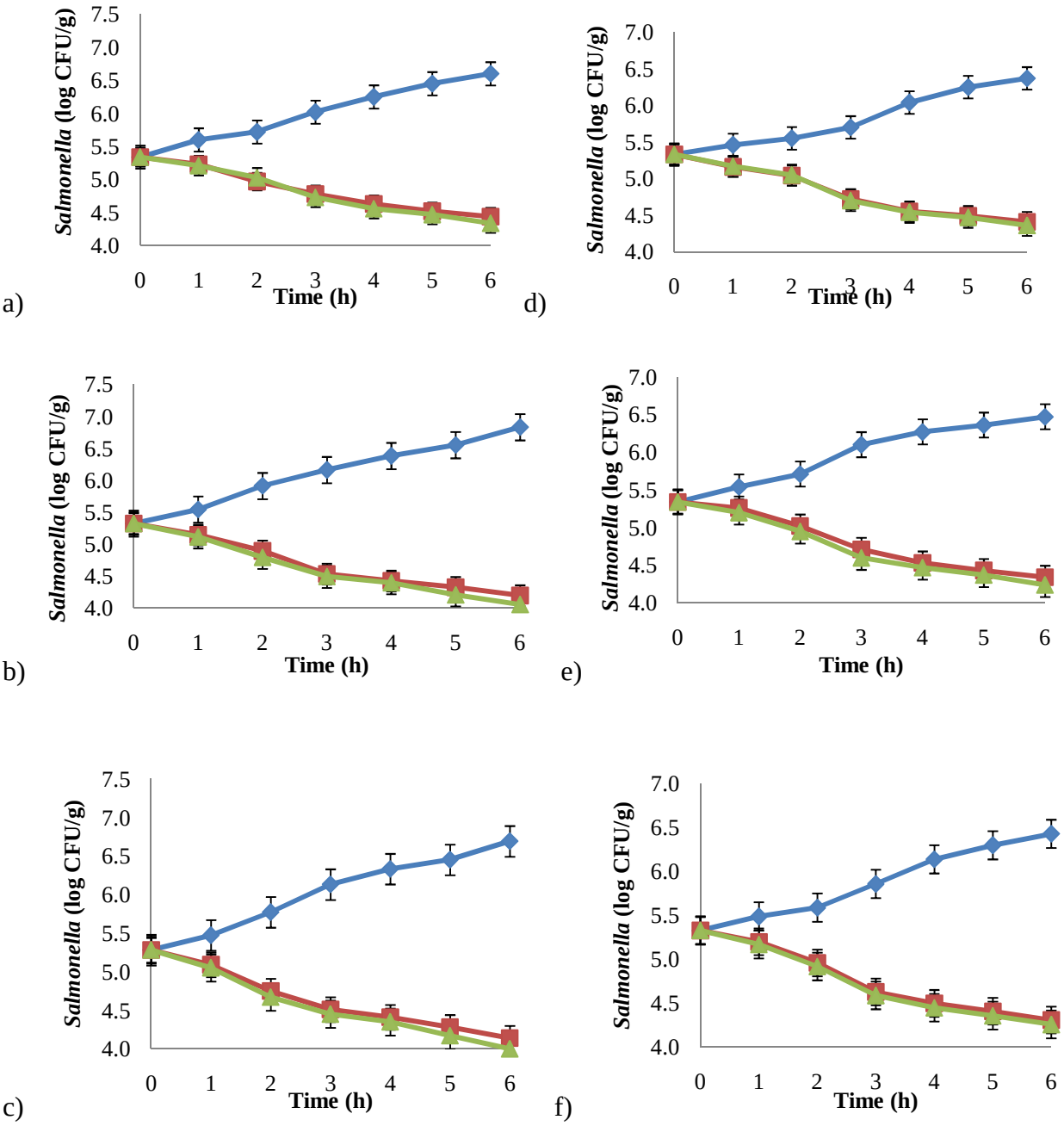

Figure 4.2

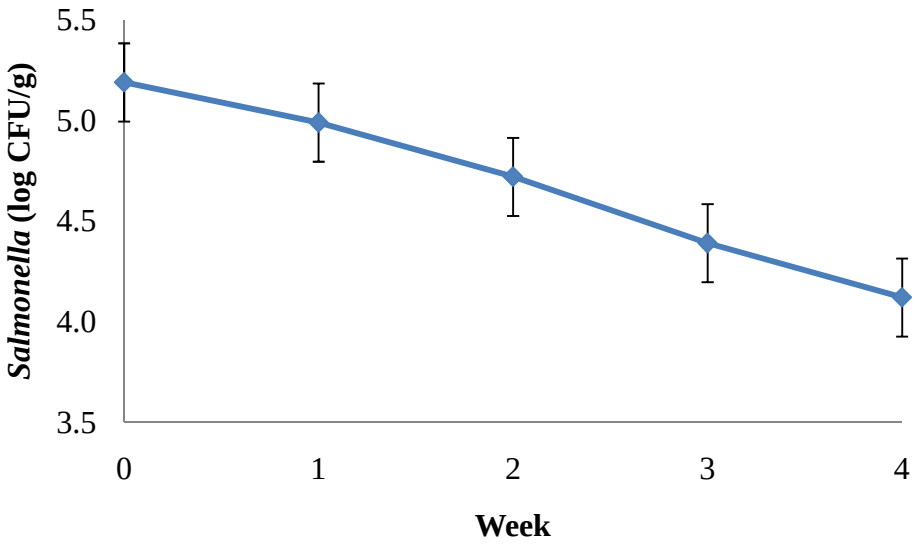

Figure 4.3

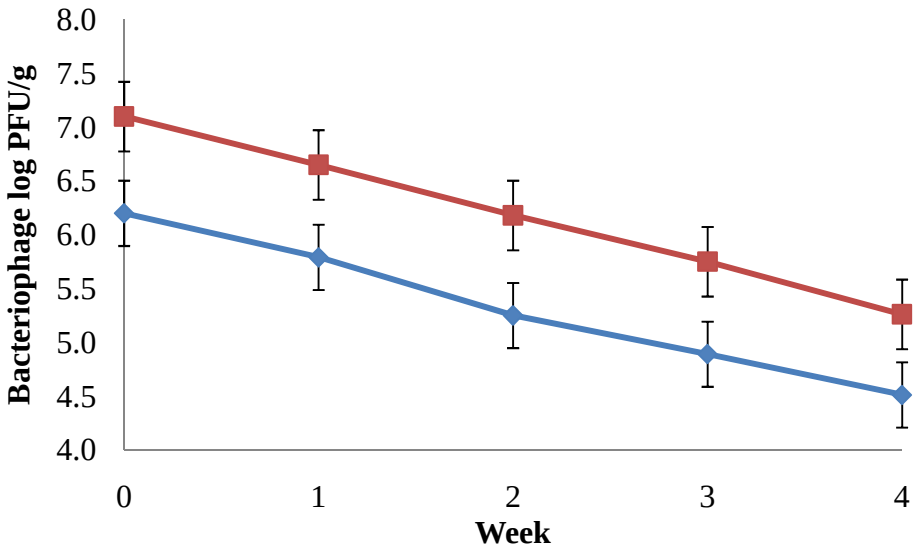

Figure 4.4

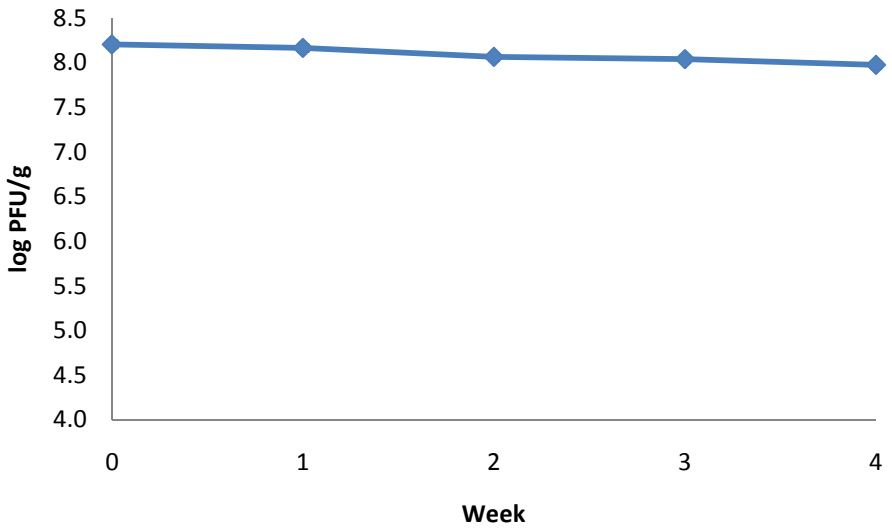

Table 4.1a. Changes in *Salmonella* populations in irradiated blood meal as affected by lyophilized bacteriophage

		Lyophilized *Salmonella*				Fresh *Salmonella*			
		MOI 10		MOI 100		MOI 10		MOI 100	
Storage (Week)	Sampling time (h)	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
0		5.27±0.20*	5.12±0.10	6.00±0.20	5.24±0.21				
1	0	4.95±0.23	5.04±0.23	4.86±0.38	4.96±0.14	4.97±0.11	5.10±0.10	5.12±0.04	4.87±0.25
	12	8.62±0.18^{A**}	7.05±0.17^{B}	8.55±0.24^{A}	6.40±0.16^{B}	9.05±0.19^{A}	7.48±0.28^{B}	8.64±0.18^{A}	6.81±0.13^{B}
2	0	4.72±0.18	4.80±0.26	4.43±0.20	4.62±0.20	5.15±0.06	4.93±0.16	4.91±0.26	5.12±0.08
	12	8.35±0.11^{A}	6.71±0.07^{B}	8.21±0.17^{A}	6.63±0.07^{B}	9.12±0.12^{A}	7.55±0.13^{B}	8.87±0.14^{A}	6.93±0.19^{B}
3	0	4.10±0.07	4.49±0.17	4.05±0.01	4.18±0.14	5.05±0.19	5.55±0.06	5.32±0.14	5.07±0.08
	12	8.47±0.30^{A}	7.31±0.16^{B}	8.42±0.21^{A}	7.02±0.14^{B}	8.76±0.22^{A}	7.61±0.11^{B}	9.02±0.07^{A}	8.22±0.11^{B}
4	0	3.94±0.16	4.02±0.44	3.80±0.18	3.93±0.07	5.36±0.15	5.43±0.22	5.17±0.15	5.21±0.22
	12	8.51±0.17^{A}	7.66±0.38^{B}	8.67±0.25^{A}	7.28±0.35^{B}	8.91±0.20^{A}	8.14±0.15^{B}	8.71±0.04^{A}	8.03±0.09^{B}

**Salmonella* log CFU/g

**Letters represent different levels of significance between the control and experimental samples among test groups

Table 4.1b. Changes in *Salmonella* populations in irradiated feather meal as affected by lyophlized bacteriophage

		Lyophilized *Salmonella*				Fresh *Salmonella*			
		MOI 10		MOI 100		MOI 10		MOI 100	
Storage (Week)	Sampling time (h)	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
0		5.07±0.20*	4.98±0.21	4.91±0.18	5.16±0.17				
1	0	5.32±0.21	4.93±0.23	4.73±0.06	4.88±0.27	5.39±0.16	4.73±0.26	5.06±0.23	4.80±0.20
	12	8.44±0.24^{A**}	6.62±0.13^{B}	8.31±0.17^{A}	6.27±0.11^{B}	8.87±0.05^{A}	7.11±0.16^{B}	8.91±0.14^{A}	7.04±0.14^{B}
2	0	4.98±0.17	4.80±0.11	4.51±0.30	4.66±0.07	4.88±0.24	5.49±0.19	5.31±0.18	4.97±0.15
	12	8.71±0.23^{A}	6.88±0.17^{B}	8.20±0.20^{A}	6.12±0.24^{B}	9.14±0.16^{A}	7.37±0.07^{B}	8.73±0.20^{A}	6.90±0.09^{B}
3	0	4.76±0.16	4.51±0.11	4.17±0.20	4.29±0.30	4.94±0.10	4.96±0.21	4.88±0.16	5.18±0.17
	12	8.55±0.23^{A}	7.22±0.04^{B}	8.27±0.30^{A}	6.89±0.03^{B}	9.03±0.03^{A}	7.19±0.14^{B}	8.80±0.15^{A}	7.96±0.06^{B}
4	0	4.37±0.35	4.10±0.10	3.97±0.21	4.04±0.08	5.04±0.11	5.35±0.28	5.11±0.33	5.22±0.27
	12	8.31±0.17^{A}	7.66±0.18^{B}	7.96±0.11^{A}	6.55±0.11^{B}	8.76±0.19^{A}	8.14±0.16^{B}	9.11±0.08^{A}	8.41±0.13^{B}

**Salmonella* log CFU/g

**Letters represent different levels of significance between the control and experimental samples among test groups

Table 4.1c. Changes in *Salmonella* populations in irradiated poultry meal as affected by lyophilized bacteriophage

		Lyophilized *Salmonella*				Fresh *Salmonella*			
		MOI 10		MOI 100		MOI 10		MOI 100	
Storage (Week)	Sampling time (h)	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
0		5.17±0.14*	5.08±0.14	5.32±0.21	5.01±0.11				
1	0	5.32±0.18	4.98±0.17	5.04±0.10	4.81±0.06	4.98±0.10	5.66±0.17	5.20±0.18	5.02±0.28
	12	7.92±0.20^{A**}	6.44±0.07^{B}	8.47±0.11^{A}	6.52±0.18^{B}	9.10±0.06^{A}	7.63±0.07^{B}	8.78±0.19^{A}	6.92±0.08^{B}
2	0	4.93±0.11	4.89±0.18	4.70±0.11	4.57±0.18	5.59±0.23	4.89±0.13	5.11±0.04	5.37±0.16
	12	7.77±0.25^{A}	6.09±0.10^{B}	8.31±0.27^{A}	6.08±0.04^{B}	8.94±0.18^{A}	7.21±0.22^{B}	8.93±0.12^{A}	7.10±0.12^{B}
3	0	4.80±0.18	4.72±0.04	4.33±0.16	4.30±0.33	5.61±0.19	4.76±0.29	4.92±0.24	5.13±0.09
	12	7.94±0.17^{A}	6.87±0.17^{B}	8.30±0.38^{A}	7.06±0.21^{B}	8.87±0.11^{A}	7.78±0.06^{B}	8.64±0.08^{A}	7.74±0.19^{B}
4	0	4.61±0.07	4.51±0.08	4.06±0.20	4.11±0.08	4.93±0.27	5.41±0.17	5.07±0.10	4.85±0.25
	12	7.66±0.04^{A}	6.93±0.08^{B}	8.19±0.24^{A}	6.72±0.25^{B}	9.03±0.08^{A}	8.17±0.14^{B}	9.09±0.11^{A}	8.26±0.16^{B}

**Salmonella* log CFU/g

**Letters represent different levels of significance between the control and experimental samples among test groups

Table 4.2a. Changes in *Salmonella* populations in irradiated blood meal as affected by fresh bacteriophage

Storage (Week)	Sampling time (h)	*Salmonella* (log CFU/g)	
		Control	Experimental
0		5.18±0.08	5.02±0.20
1	12	8.42±0.18	7.07±0.08
	24	9.26±0.24^{A*}	6.13±0.11^{B}
2	12	8.29±0.04	7.14±0.11
	24	9.07±0.10^{A}	6.22±0.23^{B}
3	12	8.41±0.14	7.39±0.21
	24	9.19±0.23^{A}	6.26±0.07^{B}
4	12	8.38±0.08	7.62±0.06
	24	8.95±0.10^{A}	6.46±0.10^{B}

**Letters represent different levels of significance between the control and experimental samples

Table 4.2b. Changes in *Salmonella* populations in irradiated feather meal as affected by fresh bacteriophage

Storage (week)	Sampling time (h)	*Salmonella* (log CFU/g)	
		Control	Experimental
0		5.17±0.17	5.08±0.13
1	12	8.28±0.07	6.96±0.11
	24	9.01±0.16^{A*}	5.97±0.13^{B}
2	12	8.50±0.17	7.24±0.13
	24	9.19±0.07^{A}	6.04±0.11^{B}
3	12	8.39±0.07	7.28±0.13
	24	9.09±0.08^{A}	6.22±0.24^{B}
4	12	8.17±0.13	7.41±0.20
	24	8.86±0.10^{A}	6.35±0.06^{B}

**Letters represent different levels of significance between the control and experimental samples

Table 4.2c. Changes in *Salmonella* populations in irradiated poultry meal as affected by fresh bacteriophage

Storage (Week)	Sampling time (h)	*Salmonella* (log CFU/g)	
		Control	Experimental
0		4.97±0.10	5.06±0.11
1	12	8.22±0.10	6.84±0.11
	24	9.10±0.11^{A*}	5.86±0.14^{B}
2	12	8.36±0.04	7.09±0.17
	24	9.27±0.04^{A}	6.17±0.16^{B}
3	12	8.16±0.11	7.17±0.07
	24	8.99±0.11^{A}	6.24±0.13^{B}
4	12	8.29±0.17	7.33±0.08
	24	9.15±0.03^{A}	6.41±0.20^{B}

**Letters represent different levels of significance between the control and experimental samples

Table 4.3. *Salmonella* enumeration in feces from mice

	Treated			Untreated	
Day	Group 1	Group 2	Group 3	Group 4	Group 5
1	BDL*	BDL	BDL	3.86±0.13**	4.79±0.13
4	BDL	BDL	BDL	3.78±0.25	4.08±0.20
8	BDL	BDL	BDL	2.85±0.11	2.78±0.04
11	BDL	BDL	BDL	3.45±0.27	3.20±0.08
15	BDL	BDL	BDL	2.60±0.06	3.00±0.13
18	BDL	BDL	BDL	BDL	BDL
22	BDL	BDL	BDL	BDL	BDL
25	BDL	BDL	BDL	BDL	BDL

* Below the detection limit 10^2 CFU/g
***Salmonella* log CFU/g

CONCLUSION

In this study we have demonstrated that finished rendered animal meals are often contaminated with pathogenic bacteria such as *Salmonella.* Through thermal tolerance testing we were able to determine the most likely cause of such contamination is post processing contamination rather than inefficient heat processing. We were able to isolate and optimize a bacteriophage cocktail that was capable of reducing *Salmonella* found on surfaces (stainless steel, rubber, HDPE plastic, and concrete) common to a rendering facility's environment as well as in the incoming raw material. On average, up to 99.9% of *Salmonella* populations were eliminated by bacteriophage treatment. By doing this, the use of a bacteriophage cocktail could potentially reduce the probability of cross-contamination, leading to no contamination in the finished meals. In addition, our results have demonstrated that a bacteriophage has the potential to be used as a feed additive. Although the stability of the lyophilized bacteriophage's in finished meals was decreased when stored at 30°C, it was improved when fresh bacteriophage was added to the feed and stored at 4°C. In addition to decreased stability, the lyophilized bacteriophage did not prevent *Salmonella* growth but did slow *Salmonella's* growth within the animal meals through 12 h. The addition of fresh bacteriophage resulted in ca. a 1 log CFU/g reduction in *Salmonella* populations within finished meal, suggesting the bacteriophage's potential as a feed additive is increased when using a fresh bacteriophage suspension. The effectiveness of bacteriophage as a feed additive was further confirmed during an animal trial. Mice receiving bacteriophage in their feed showed no signs of infection following

Salmonella inoculation. In conclusion, we have demonstrated that a bacteriophage cocktail can be used to reduce the risk of *Salmonella* contamination in the processing environment, the feed, and upon feed consumption.