

ABSTRACT

Stam, Christina Nicole. Prevalence and Persistence of Select Foodborne Pathogens in a mid-Atlantic Turkey Processing Facility. (Under the direction of Dr. Lee-Ann Jaykus).

Listeria monocytogenes, *Salmonella* and *Campylobacter* combined are responsible for the majority of foodborne disease hospitalizations and over 1200 deaths annually in the U.S. alone. Although raw poultry has been identified as a source of these pathogens, most microbiological studies have focused on broilers with little attention given to turkey processing. The purpose of this research was to investigate the prevalence of select pathogens (*L. monocytogenes*, *Salmonella* spp., and *Campylobacter* spp.) and microbiological indicators (*Enterococcus* spp.) in the turkey processing environment. Environmental samples were collected in one Southeastern processing facility using swab methods at two month intervals over a period of 14 months. Samples were taken from conveyors, drains, walls and various food contact surfaces. Isolation and identification of bacteria was done using the USDA-FSIS Microbiology Laboratory Guidebook protocols. The prevalence of contamination was 11.5%, 7.4%, and 0.4% for *L. monocytogenes*, *Salmonella*, and *Campylobacter*, respectively. *Enterococcus* spp., an environmental indicator of fecal contamination, were isolated from over >75% of the samples screened. *Salmonella* isolates were typed using pulsed-field gel electrophoresis (PFGE) and *Enterococcus* isolates were speciated by PCR with antibiotic resistance profiles characterized using the SensiTitre system. A diverse set of relatively non-persistent *Salmonella* strains were obtained from the processing environment, as evaluated by PFGE. Thirty-nine percent of the *Enterococcus* isolates were speciated as *E. faecium* and 55% were *E. faecalis*. Both *E. faecalis* as *E. faecium* strains were

susceptible to most antibiotics of human clinical relevance. Thirty-three *L. monocytogenes* strains were screened for their biofilm formation capabilities using a microtiter well assay. None of the strains formed a biofilm in monoculture; however, sixteen of the strains were able to form a biofilm in the presence of another organism. Data collected in prevalence studies such as this one can help processors identify contamination frequency and sites in an effort to control resident pathogenic bacteria in the processing environment.

**PREVALENCE AND PERSISTENCE OF SELECT FOODBORNE PATHOGENS
IN A MID-ATLANTIC TURKEY PROCESSING FACILITY**

By

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CHAPTER 1

Literature Review

Public Health Implications of Foodborne Disease

It is estimated that 76 million illnesses a year are caused by foodborne pathogens (Mead et al., 1999). Of those, 14 million illnesses are from known pathogens (Mead et al., 1999), with the bacterial pathogens of greatest concern being *Listeria monocytogenes*, *Campylobacter* spp. and *Salmonella* spp.

(<http://www.ers.usda.gov/Briefing/FoodborneDisease/foodandpathogens/>). *Listeria monocytogenes* and *Salmonella* account for approximately 80% of all deaths from known foodborne disease agents (Mead et al., 1999). In 1999 alone, all three microorganisms combined were responsible for most of the foodborne disease hospitalizations and over 1200 deaths in the U.S. (Mead et al., 1999). The economic impact of illness associated with *L. monocytogenes*, *Campylobacter* and *Salmonella* has been estimated at 5.9 billion a year (<http://www.ers.usda.gov/Briefing/FoodborneDisease/features.htm>). This figure includes medical costs, productivity losses and costs associated with premature deaths (<http://www.ers.usda.gov/Briefing/FoodborneDisease/features.htm>). Interestingly, since the onset of the CDC FoodNet program, the incidence of listeriosis and *Campylobacteriosis* appear to be steadily decreasing, while the incidence of *Salmonella* infections has continued to increase over the 3 year period from 2000 to 2002 (Anonymous, 2003; Anonymous, 2002; Anonymous, 2001) (Table 1).

HACCP and Performance Standards

HACCP

In 1996, the federal government passed the Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) Systems final rule (USDA, 1996). The purpose of the HACCP rule was to provide a series of preventive controls based on 7 principles (USDA, 1996; Rose et al., 2002; McNarmara, 1997; Anonymous, 1999). These 7 principles include: (i) conducting a hazard analysis to determine where chemical, biological and physical hazards occur in a process; (ii) establishing critical control points (CCP's) that identify where a food safety hazard can best be controlled; (iii) setting critical limits to determine when a CCP is no longer in control and becomes a food safety hazard; (iv) monitoring CCP's to ensure that they stay within the critical limit; (v) establishing corrective actions when CCP's breach the critical limit; (vi) keeping records to ensure compliance; and (vii) verification to ensure that the HACCP plan is working correctly (USDA, 1996; Anonymous, 1999) (Figure 1).

As of January 1998, all meat and poultry facilities with greater than 500 employees were required to implement HACCP, and therefore be in compliance with the final rule (USDA, 1996; Rose et al., 2002). In 1999 and 2000, the small and very small establishments were required to be compliant, respectively (USDA, 1996). In addition to a USDA-FSIS approved HACCP plan, regulations require that each facility also have written sanitation standard operating procedures (SSOP's), regular microbiological testing for generic *E.coli*, and compliance with the *Salmonella* performance standards (USDA, 1996; Rose et al., 2002).

Performance Standards

In response to epidemiological data and concern over *Salmonella* contamination in poultry, pathogen performance standards were established for *Salmonella* because (i) *Salmonella* was considered one of the two common causes of bacterial foodborne illness; (ii) *Salmonella* can colonize a variety of mammals and birds at high frequency; (iii) *Salmonella* can be recovered from a variety of meat and poultry products; and (iv) reduction of fecal contamination should be effective at reducing both *Salmonella* and other foodborne pathogens of enteric origin (USDA, 1996). The initial baseline performance standards for *Salmonella* in broilers was a prevalence of contamination of 20%; for ground chicken, prevalence was 44.6%; and for ground turkey, 49.9% (Rose et al., 2002). These percentages were based on a nationwide study and represent the maximum allowable *Salmonella* prevalence in a given sample set (Rose et al., 2002). The FSIS has determined that more than 80% of the facilities met the established *Salmonella* performance standards within the first 3 years of regulatory implementation, which started in 1998 (Rose et al., 2002). For instance, in 1998, 90.9% of broilers and 85.7% of ground turkey passed the performance standards, but by 2000 the percentage passing the performance standards increased to 91.6% for broilers, 88.2% for ground turkey and 100% for ground chicken (Rose et al., 2002). *E.coli* performance standards were established because the organism is an indicator of fecal contamination and the potential for contamination with enteric pathogens (USDA, 1996). These standards require all processing facilities to test for generic *E.coli* (Rose et al., 2002). The maximum allowable limit is 10^3 CFU/ml of carcass rinse in chickens, 10^4 CFU/ml in

swine and 10^2 CFU/ml in cattle carcasses (USDA, 1996); also, 3 out of 13 samples can test positive for the presence of generic *E.coli* (USDA, 1996).

Role of Poultry in Foodborne Disease

Pathogens

Poultry and poultry products are a common vehicle of foodborne illness (Uyttendaele et al., 1999). Ten to 18.5% of all reported foodborne disease outbreaks are associated with poultry, with turkey responsible for 56% and chickens responsible for 44% of these (Bryan and Doyle, 1995; Coleman et al., 2003). The pathogens most commonly associated with poultry include *Salmonella* spp., *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* (Uyttendaele et al., 1999). Of the poultry associated foodborne disease outbreaks reported between 1992 and 1996, 67.3% were caused by *Salmonella* (Bryan and Doyle, 1995; Panisello et al., 2000). Per capita consumption of poultry products has been increasing (Brewer et al., 1995; Wallace et al., 1998), and the frequently cited factors contributing to poultry-associated foodborne disease continue to be improper handling and cooking of raw meat (Whyte et al., 2002; Stern et al., 2001).

Prevalence of Contamination

Poultry animals are asymptomatic carriers of *Salmonella* and *Campylobacter* spp. (Fluckey et al., 2003; Stern et al., 2001). Initial contamination occurs at the pre-harvest level, with survival and propagation of microorganisms in farms, processing plants and cooking operations (Bryan and Doyle, 1995). In many cases, animals that are free of contamination may subsequently become contaminated during slaughter, evisceration and

processing (Wallace et al., 1998). It is believed that technological changes associated with the mass production and processing of poultry have exacerbated these food safety issues (Fluckey et al., 2003).

Salmonella and *Campylobacter* are found in the intestinal tracts of birds, and appear to be acquired from feed and other environmental sources, with stress an exacerbating factor (Fluckey et al., 2003; Bryan and Doyle, 1995). *C. jejuni* can colonize chicks, making it readily transmissible through the entire flock (Stern et al., 1988; Saleha et al., 1998). Only 3-4% of all flocks entering the processing plant are positive for *Salmonella*, but 87.6% of the flocks are positive for *Campylobacter* (Stern et al., 2001; Sanchez et al., 2002). *Campylobacter* is usually found in higher amounts than *Salmonella* on carcasses as well (Sanchez et al., 2002). During processing, there is no significant reduction in the numbers of *Salmonella* or *Campylobacter*, making these pathogens difficult to eliminate (Fluckey et al., 2003). *S. enterica* serovar Enteritidis is more prevalent on chicken carcasses and *Campylobacter* is more prevalent in turkeys (Wallace et al., 1998; Uyttendaele et al., 1998). For turkey carcasses, *Campylobacter* is usually present on 90% of carcasses and *Salmonella* on 2.6% (Zhao et al., 2001; Lam et al., 1992). Approximately 1.2% of turkeys and 2.2% of broiler flocks are contaminated with both *C. jejuni/coli* and *Salmonella* (Harns et al., 1986; Wedderkipp et al., 2001).

L. monocytogenes has not been a major contaminant of raw turkey meat, however this may be due to a general lack of data. *L. monocytogenes*, is, however, frequently found in other sources of poultry meat (Uyttendaele et al., 1997; Ojeniyi et al., 1996). For instance, raw chickens may be contaminated with *L. monocytogenes* at a prevalence of 15-32% (Capita et al., 2001; Franco et al., 1995). In a study of retail oven-ready

chickens, *L. monocytogenes* was isolated from 50-66% of all birds tested (Geornaras et al., 1995; Ojeniyi et al., 1996). Separated chicken pieces, including the skin of drumsticks and wings, seem to have the highest level of contamination, presumably due to increased contact with processing equipment and manual handling (Geornaras and VonHoly, 2000; Franco et al., 1995). *L. monocytogenes* is usually detected on carcasses after evisceration, suggesting that environmental conditions during processing, rather than the bird itself, is the source of the organism (Geornaras et al., 1995; Ojeniyi et al., 1996).

Prevalence studies at the retail level show widely different contamination rates, depending upon commodity and study location. For example, 50-90% of broilers and turkeys were reportedly contaminated with *Campylobacter* at the retail level (Federighi et al., 1999; Sanchez et al., 2002; Lam et al., 1992). A survey done in Washington D.C. reported that 70.7% of chickens and 14% of turkeys were positive for *Campylobacter* (Zhao et al., 2001). In Japan, 67.9% of chickens were positive for *Campylobacter* and 24.1% were positive for *Salmonella* (Tokumaro et al, 1990). However, in Belgian retail markets, *L. monocytogenes* was found more frequently on poultry, with contamination rates of 38.2%, while the prevalence of contamination with *Salmonella* and *C. jejuni/coli* was 36.5% and 28.5%, respectively (Uyttendaele et al., 1999).

The Significance of *Enterococcus* spp.

Enterococcus spp. are ubiquitous to the environment and have been isolated from soil and water. They are frequently found in clinical settings and are common inhabitants of the gastrointestinal tracts of humans and animals (Johnston and Jaykus, 2004). Due to their ubiquitous nature and recent clinical disease associated with this genus,

Enterococcus spp. have become a concern in the United States. In particular, multiple-drug-resistant *E. faecalis* and *E. faecium* are the third leading cause of all nosocomial infections in intensive care units (Johnston and Jaykus, 2004; Eaton and Gasson, 2002; Hayes et al., 2004). Historically, *E. faecalis* has been the dominant causative agent of these infections, but a recent increase in *E. faecium* infections is believed to be due to the emergence of vancomycin-resistance enterococci (VRE) in food animal production (Eaton and Gasson, 2002; Hayes et al., 2004). In the European Union, VRE have been found in broilers and pigs, and has been linked to the use of a human antimicrobial glycopeptide, avoparcin, as a growth promoter in animal production (Manson et al., 2004; Hayes et al., 2004). Avoparcin use in animal production is thought to be responsible primarily for nonhospitalized increases in VRE infections in the European Union (Hayes et al., 2004).

In response, the European Union has created bans on avoparcin use; since these bans, Denmark has been the only EU country to see a decrease in prevalence of VRE; Norway for instance, still had VRE present in broilers after 5 years of discontinued avoparcin use (Manson et al., 2004). Consequently, continued concern over growing antimicrobial resistance has led the EU to ban all human antimicrobials as growth promoters for animals (Hayes et al., 2004). Concern in the United States over the use of human antimicrobials in animal production has been growing, in large part because most antimicrobials in animal production are analogues of the antibiotics used to treat human infections, in particular the VRE (Hayes et al., 2004). However, the US has not been as aggressive as the European Union in banning the use of these antibiotics in animal

production. To date, the actual importance of meat products, including poultry, to the evolution and transmission of VRE is unknown.

Biofilms

To maintain viability and growth, microorganisms seek surfaces that have been conditioned with nutrients that can promote their survival (Zottola, 1994; Kumar and Anand, 1998). Once the microorganisms multiply and colonize, they attach to the surface and form a biofilm (Donlan, 2002; Kumar and Anand, 1998; Zottola, 2001). A biofilm is a functional consortium of microbial cells that adhere to a wet surface and become immobilized in a protective polysaccharide matrix that can entrap nutrients and other microbes, allowing for subsequent microbial growth (Zottola, 2001; Donlan, 2002; Kumar and Anand, 1998; Zottola, 1994; Lindsay et al., 1996; Arnold and Silvers, 2000; Prigent-Combaret et al., 2000). The purpose of the biofilm is to provide the bacteria protection against adverse conditions in the environment such as toxicities, sanitizers, antibiotics, predators, desiccation and mechanical damage (Lindsay et al., 1996; Lee Wong, 1998; Watnick and Kolter, 1999).

Biofilms in Food Processing

Attachment of microorganisms to food contact surfaces can impact the food industry economically, and through associated health risks (Austin et al., 1998). Pathogenic and spoilage bacteria have been shown to attach to a wide variety of food-contact and non-contact surfaces (Lindsay et al., 1996; LeeWong, 1998). These surfaces include glass, stainless steel, rubber, plastics, aluminum, Teflon, Buna-N (which is found in dairy plants), rubber “fingers” (used in chicken processing), floor drains and crevices in floors, walls and equipment found through out a processing facility (Lindsay et al.,

1996; Kumar and Anand, 1998; Zottola, 2001). The areas of the equipment most prone to biofilm formation include dead ends, joints, valves and gaskets (LeeWong, 1998). Raw materials and ingredients have also been studied as the source of biofilm development (Kumar and Anand, 1998). For instance, attached bacteria have been found in the collagen fibers of raw beef, pork and lamb, as well as on the skin surface of poultry (Zottola and Sasahara, 1994; Hood and Zottola, 1997; Arnold and Bailey, 2000). Attached bacteria can enter the food supply by sloughing off from the biofilm during processing or sanitizing (Zottola and Sasahara, 1994). Contamination from biofilms is sporadic, as cells do not continuously slough, making control or determination of the source of the contaminant(s) more challenging (Zottola, 2001). If the cause of the contamination is spoilage bacteria, it can impact the processor by reducing product shelf life with associated economic losses (Kumar and Anand, 1998). Contamination from pathogenic microorganisms can have a major public health impact (Austin et al., 1998). *E. coli* O157:H7, *Salmonella enterica* serovar. Enteritidis, *L. monocytogenes*, and *C. jejuni* have been all found to adhere to a variety of surfaces and have been resistant to sanitizers (LeeWong, 1998; Trachoo et al., 2002; Beresford et al., 2001; Joseph et al., 2001; Bonafonte et al., 2000).

Proper cleaning and sanitizing is the main element in controlling and removing biofilms (Zottola, 2001.). In one study, several biofilms consisting of various *Listeria* spp. were found to be resistant to chlorine at the highest levels approved by the USDA for use in the food industry (Arnold and Silvers, 2000). In Canada, an outbreak of *Salmonella* occurred because routine sanitation procedures were ineffective at removing the bacteria in a biofilm (Austin et al., 1998).

Formation of Biofilms

Microorganisms form biofilms to protect themselves against adverse conditions (Lindsay et al., 1996; LeeWong, 1998). These biofilms can be comprised of single or mixed species (Kumar and Anand, 1998), although mixed species biofilms offer added protection because they are more stable and create a larger and thicker biofilm mass (Donlan, 2002). There are five stages associated with the formation and development of biofilms (Kumar and Anand, 1998; Zottola, 1994; Lindsay et al., 1996). These steps include the following: (i) transport of nutrients to a surface; (ii) surface conditioning of films; (iii) attachment of microorganisms; (iv) metabolism and growth; and finally (v) detachment and dispersal. These phases will be discussed in greater detail below.

Transport of Nutrients: Nutrients comprised of organic and inorganic molecules are transported to a surface that will promote and aid in the growth of bacteria (Zottola, 2001; Zottola, 1994). The nutrients become free-floating particles due to aerosolization during cleaning and ultimately come into contact with a solid surface and sediment, providing a substratum to allow biologically active organisms to adhere (Zottola and Sasahara, 1994).

Conditioning Films: The organic molecules covering the surface lead to a situation called “conditioning” (Zottola and Sasahara, 1994). A conditioning film is formed when the bacteria, along with the accompanying nutrients, get adsorbed to a surface that has been coated with polymers from an aqueous medium (Donlan, 2002; Kumar and Anand, 1998). In food processing, a conditioning film can be comprised of proteins from milk or meat, or any other food – related matrix that has a high nutrient concentration (Kumar and Anand, 1998; Zottola and Sasahara, 1994). The purpose of the

conditioning film is not only to provide nutrients, which aid in microbial growth, but also to alter the physico-chemical properties of the surface so that the surface is conducive to microbial attachment (Kumar and Anand, 1998).

Attachment: For a bacterium to attach to a surface it first must overcome an electrostatic repulsion barrier (Donlan, 2002;). Once the cell is able to get less than 1 nm from the surface, a strong attraction occurs between the surface and the bacterium, resulting in attachment (Watnick and Kolter, 1999). The initial bacterial adhesion to the surface is reversible and based on weak interactions of electrostatic attraction and Van der Waals forces (Zottola, 1994; Beresford et al., 2001; Hood and Zottola, 1997). Due to the attractive forces and Brownian motion exhibited by the bacteria, the cells can easily be removed from the surface (Kumar and Anand, 1998). At this point, product contamination can occur more readily because the bacteria are not irreversibly attached to the surface (Arnold and Bailey, 2000).

The length of time a cell remains attached to the surface is dependent on surface charge, pH, nutrient levels, contact time and hydrophobic interactions between cell surface polymers, fimbriae and proteins (Zottola and Sasahara, 1994; Donlan, 2002; Arnold and Silvers, 2000). For instance, curli fimbriae associated with *E.coli* promote bacterial attachment to the substratum, as well as aiding cell-to-cell contact with other microorganisms (Prigent-Combaret et al., 2000). Proteins can also favor or inhibit the attachment of bacteria (Kumar and Anand, 1998; Lindsay et al., 1996). In a study of biofilm formation by *L. monocytogenes* and *Salmonella typhimurium*, attachment to stainless steel was inhibited by milk proteins (Hood and Zottola, 1997). Rough surfaces,

particularly cracks and crevices that might be associated with worn stainless steel, may also promote bacterial attachment (Donlan, 2002; Zottola and Sasahara, 1994).

Metabolism and Growth: During metabolism and growth, the cells go from reversible to irreversible attachment, which is caused by the formation of extracellular polymeric substances (EPS) (Beresford et al., 2001). EPS is the most abundant material in biofilms and is comprised of polysaccharides and protein (Donlan, 2002; Hood and Zottola, 1997). EPS is produced by the microcolonies, creating a slime-like layer that binds cells to the surface, protecting and stabilizing them from the environment (Kumar and Anand, 1998; Ramesh et al., 2002; Zottola and Sasahara, 1994). The binding force created by the EPS is due to the ability of calcium and magnesium to crosslink in the polymer strands (Donlan, 2002).

Growth of microcolonies in the biofilm usually occurs due to nutrient utilization (Kumar and Anand, 1998). In two different studies of *Salmonella* serovar. *Enteritidis*, the formation of a biofilm and its subsequent thickness was due in part to excess glucose in the medium as well as aggregative fimbriae (Bonafonte et al., 2000; Austin et al., 1998). However, nutrients are not always necessary for growth, as *V. cholerae* can form a biofilm on abiotic surfaces void of nutrients (Watnick and Kolter, 1999). Water channels in the biofilm are essential to bacterial cell survival because they permit nutrients to flow into the biofilm, while promoting the removal of waste products (DeKievit and Iglewski, 2000). Water channels also allow for separation of species in mixed biofilms (Donlan, 2002;) and they protect the bacteria from dehydration (Kumar and Anand, 1998).

In one study, *L. monocytogenes* was found to grow only in the presence of an EPS-producing microorganism such as *P. fragi* (Zottola and Sasahara, 1994). This was believed to be due in part to quorum sensing, where the bacteria behave collectively and form a biofilm to protect themselves and each other from the environment (DeKievit and Iglewski, 2000). Quorum sensing is a form of intracellular communication facilitated by molecular signals called autoinducers (DeKievit and Iglewski, 2000). When the signals reach a threshold concentration, they activate or repress the other organisms' genes to allow for symbiotic behavior (Donlan, 2002;). The cell-to-cell signaling also allows for the bacteria to take on different behaviors (HaleBoothe et al., 1999). Quorum sensing is a fruitful area of further research and there is much to learn about its role in biofilm formation.

Detachment and Dispersal: Detachment of biofilms is caused by physical forces of shearing, sloughing and abrasion (Donlan, 2002). Shearing is caused from flow effects that continuously remove parts of the biofilm (Donlan, 2002). Sloughing is sporadic and caused by cells at the edge of the biofilm that break off during routine cleaning (Zottola and Sasahara, 1994; Kumar and Anand, 1998). In a study by Austin et al (1998), serovar. *Enteritidis* was easily sloughed from Teflon and stainless steel surfaces due to the combined effects of cell clumping and lack of fimbriae (Austin et al., 1998). Dispersal of the biofilms is a consequence of detachment, which leads to parts of the biofilm contaminating food products (Donlan, 2002; Hood and Zottola, 1997). Biofilm pieces detach periodically from actively growing daughter cells, making contamination by spoilage or pathogenic organisms sporadic and not continuous (Zottola, 2001; Donlan, 2002).

Sanitation and Control of Biofilms

Attached microorganisms are generally more resistant to sanitation chemicals than are their detached counterparts (LeeWong, 1998; Arnold and Silvers, 2000). This resistance is due to protection from organic materials and the EPS layer, which prevents chemicals from entering the biofilm or causes inactivation of the sanitizer (Donlan, 2002; Lindsay et al., 1996; Ramesh et al., 2002). Nutrient deficiency and the length of time the biofilm has been established will also affect resistance to sanitation (Kumar and Anand, 1998; Zottola, 1994). The surface may also influence the biofilm resistance because difficulties encountered when cleaning rough surfaces or non-food contact surfaces may allow for rapid biofilm reformation and/or continued growth (LeeWong, 1998; Bos et al., 1999; Zottola and Sasahara, 1994).

High temperatures, halogens, chlorine, iodine, quaternary ammonium compounds and acids are all used as sanitizers in food processing (Zottola and Sasahara, 1994). Those chemicals used for clean in place (CIP) procedures can still leave attached bacteria (LeeWong, 1998). In some instances, there is conflicting data regarding the efficacy of certain sanitizers in controlling biofilms. For example, cellulose associated with serovar *Enteritidis* biofilms correlated with chlorine resistance at approved concentrations for use (Solano et al., 2002). However, in another study, 500ppm of sodium hypochlorite and sodium chlorite with alkaline peroxide effectively eliminated *Salmonella* biofilms within 2 minutes (Ramesh et al., 2002).

Frequent cleaning is essential in preventing bacterial attachment (Zottola and Sasahara, 1994). Proper cleaning involves using a detergent to first loosen the “soil”, which is covering the biofilm (Zottola and Sasahara, 1994; Zottola, 1994). In this case,

the soil is composed of organic materials: fats, carbohydrates, proteins and minerals, all of which can inactivate the sanitizer (Zottola, 1994). After the soil is removed, a sanitizer can be used because the biofilm is no longer protected, so the sanitizer can disrupt the polysaccharide matrix (Kumar and Anand, 1998; Zottola and Sasahara, 1994; Zottola, 1994). For example, removal of necessary substrates that are rapidly metabolized is essential in controlling biofilm growth in poultry processing (HaleBoothe et al., 1999; HaleBoothe and Arnold, 2002).

There are several chemical mechanisms for controlling biofilm formation. Preventing the synthesis of EPS (Solano et al., 2002), which can be accomplished by the use of specific enzymes, is feasible but must be specific to the target biofilm microbiota (Kumar and Anand, 1998). In some instances, bacteriocins have been shown to inhibit bacterial attachment by exhibiting bactericidal properties (Kumar and Anand, 1998). For example, nisin effectively prevented surface growth of *L. monocytogenes* when the surface was adsorbed with the bacteriocin (Kumar and Anand, 1998). Electrochemical processes, such as electropolishing of stainless steel, results in the surface removal of metal ions, which reduces electrostatic or hydrophobic interactions, making surfaces less susceptible to bacterial attachment (Arnold and Bailey, 2000).

Various physical means to control biofilm formation are also being investigated. These include the use of electrical currents, magnetic fields and ultrasound treatments (Kumar and Anand, 1998). The use of electrical currents increases the transfer of lethal concentrations of antibiotics into the biofilm matrix by penetrating cell target sites more quickly (Kumar and Anand, 1998). Magnetic fields are more effective at higher temperatures and appear to affect the bacterial membrane structure and intracellular water

activity (Okuno et al., 1993). Ultrasound treatments have been used with antibiotics to speed up penetration of the antibiotics within the biofilm bacteria (Carmen et al., 2004).

Methods for Detecting and Studying Biofilms

Two methods are commonly used to screen bacterial strains for their potential as biofilm producers. As such, these are designated (i) indirect and (ii) direct methods (Djordjevic et al., 2002). For direct methods, the biofilm is actually produced on the surface of interest, and its integrity examined using microscopic methods. In the indirect approach, biofilms are produced on surfaces of interest, subsequently detached, and their formation evaluated indirectly by monitoring cell density. In this case, high cell density would suggest that a particular strain was also a good biofilm producer (Djordjevic et al., 2002). One common method involves inoculating a coupon or tube comprised of a specific material, such as stainless steel, plastic, rubber or some other material commonly used in the food-processing environment, and then using a direct method to assess growth (Beresford et al., 2001). The coupons and tubes are allowed to incubate for a select time with or without agitation (Hood and Zottola, 1997). After incubation, coupons are washed to remove any unattached cells (Beresford et al., 2001). Assessment of biofilm growth is directly measured through microscopy techniques, such as epifluorescence light and confocal scanning laser microscopy, to name a few (Kalmokoff et al., 2001; Chae and Schraft, 2000).

The microtiter plate assay has also been used in assessing biofilm formation. It is an indirect measurement that involves growing the bacteria in 200ul wells and reading the optical density after unattached bacteria have been removed and attached bacteria stained (Djordjevic et al., 2002). The microtiter assay does not involve a direct

measurement of biofilm development, but rather uses cellular growth rates and final cell density as an indicator of biofilm formation capability (Djordjevic et al, 2002).

Representative studies of biofilm production among strains of *Listeria monocytogenes* are described in Table 2.

Microbiological Strain Typing

In microbiology, two general approaches are used to determine strain relatedness: phenotypic and genotypic methods (Farber, 1996). Phenotypic methods, including serotyping, biotyping, and bacteriophage typing, are used to determine if strains are related by virtue of phenotypic characteristics such as sugar utilization or surface antigens (Farber, 1996). The main advantage to genotyping is that it can discriminate between closely related strains (Farber, 1996). PCR typing, ribotyping, plasmid typing and pulsed-field gel electrophoresis (PFGE) are several methods commonly used to discriminate between organisms based on genetic relatedness (Farber, 1996).

Pulsed field gel electrophoresis (PFGE)

PFGE is a genotyping method that uses alternating electric fields to separate DNA molecules as large as 12 Mb (Maule, 1998; Wrestler, 1996). The banding patterns of various strains have been used for studying the size and shape of microbial genomes, cloning, and karyotyping of yeasts, fungi and protozoa (Maule, 1998). From a food safety standpoint, the most common use of PFGE has been to study disease outbreaks of various bacteria, including *Salmonella*, *Campylobacter*, *Shigella sonnei*, *Enterococcus faecalis*, *Vibrio cholerae* O1 and *Escherichia coli* O114 (Nadeau et al., 2003; Murase et al., 1995). Particularly in discriminating *Salmonella* strains, PFGE is considered the “gold standard” molecular typing method (Kotetishvili et al., 2002).

Instrumentation for PFGE

Contour-clamped homogeneous electric field (CHEF) is the most popular PFGE system (Wrestler, 1996). The CHEF system uses a hexagonal electrode array that can easily be programmed to the specific angle and pulse times (Maule, 1998). The pulses switch through a range of 120° angles that will separate large DNA molecules with the small reorientation angles (Bustamante et al., 1993; Wrestler, 1996). The changing of angles of electric current and pulse times causes DNA to move in a zigzag motion through the gel (Wrestler, 1996; Farber, 1996). The slow migration speed of large molecules creates a longer aligning time when compared to smaller molecules (Wrestler, 1996). The size of the resolved fragments is determined by the pulse switch times; the smallest DNA fragment is controlled by the initial switch time and the largest fragment by the final switch time (Maule, 1998; Wrestler, 1996). A cooled recirculating buffer provides uniform pH of the gel, providing even discrimination of DNA banding patterns (Maule, 1998).

Sample Preparation for PFGE: Large DNA fragments are subject to shearing and need to be protected during electrophoresis (Farber, 1996). Therefore, DNA is embedded in low-melt temperature agarose plugs that protect the large DNA molecules by encapsulation (Maule, 1998). Restriction enzymes are needed to modify the DNA to produce simple profiles of 10-20 bands (Graves and Swaminathan, 2001; Maule, 1998). The enzymes used, called infrequent cutters, typically recognize anywhere from 4-8 nucleotide bases (Farber, 1996). Large DNA of 20-200kb requires lambda concatemers, which is why a lambda ladder is used to measure the fragment size when running a gel (Maule, 1998). When comparing PFGE patterns, it is difficult to compare DNA cut with

different restriction enzymes because of variation in patterns. For PFGE applications to *Salmonella*, the restriction enzymes *Xba*I and *Spe*I are the most commonly used (Fernandez et al., 2003).

Interpreting PFGE: Accurate comparison of PFGE patterns depends on having at least 10 fragments to interpret (Tenover et al., 1995). When determining pattern relatedness, a scale of variations is used. In general, variation in 2-3 bands means that the strains are closely related; variations in 4-6 bands suggests that the strains are possibly related; and variations greater than 7 means the strains are different (Farber, 1996). In outbreak investigation, variation in a band or two is common and could be caused by insertions, deletions, or random point mutations (Laconcha et al., 1998).

Applications of PFGE: Early uses of PFGE for *Salmonella* strain typing focused almost exclusively on outbreak investigation. For instance, *Salmonella* banding patterns produced by the restriction enzyme *Bln*I were used to identify a European outbreak as the initial source of a *Salmonella* strain associated with a second outbreak in Chile (Fernandez et al., 2003). Later applications focused on tracking strains through the environment. For example, PFGE patterns were used to study the source of *Salmonella* on a swine farm, where similar banding patterns suggested a connection between rodents, birds and the environment (Sandvang et al., 2000). Currently, similar yet unrelated patterns are being studied to evaluate relatedness between concurrent and/or epidemic strains (Tsen et al., 2000). Table 3 summarizes recent applications of PFGE to *Salmonella* strain typing.

Advantages and Disadvantages to PFGE

PFGE has become an invaluable tool because typing and discrimination of strains requires minimal band fragments, meaning that interpretation is relatively simple (Garaizar et al., 2000). Once a strain is typed, its pattern will remain stable and can be duplicated for up to several years (Liesegang et al., 2002). In a study of 55 *S. typhi* strains, PFGE typing using the *XbaI* enzyme produced 41 unique banding patterns, demonstrating that the technique can be highly discriminatory (Tsen et al., 2000). Furthermore, isolates indistinguishable by PFGE do not typically show different results when other methods are used (Sandvang et al., 2000).

However, PFGE is a time-consuming process and more rapid typing methods are available (Graves and Swaminathan, 2001; Laconcha et al., 2000). Lack of a standardized protocol has created variability between results produced in separate labs, although this is less of a problem today (Kotetishvili et al., 2002). Standards for interpreting the patterns and the use of previously designated enzymes will help alleviate such inconsistency among results (Tenover et al., 1995)

Table 1. Public Health implications of select foodborne pathogens

Pathogens	Disease Incidence*			Total** Hospitalizations	Total** Deaths	Costs° (2000 In Billions)	Common Vehicles of Transmission
	'00	'01	'02				
<i>Salmonella</i>	14.4	15.1	16.10	16,430	582	2.4	Poultry, meat, eggs, milk
<i>Campylobacter</i>	15.7	13.8	13.37	13,174	124	1.2	Poultry, milk, water, cheese, pork, hamburger
<i>Listeria monocytogenes</i>	0.3	0.3	0.27	2,322	504	2.3	Soft cheese, pate, ground meat, poultry, dairy products

*Incidence '01 Per 100,000 persons from 9 sites (MMWR, 2002)

*Incidence '02 Per 100,000 persons from 9 sites (MMWR, 2003)

** Estimated Annual Hospitalizations and Deaths (Mead et al, 1999)

°Costs ERS/USDA web page (<http://www.ers.usda.gov/Briefing/FoodborneDisease/>)

Table 2. Methods of *L. monocytogenes* biofilms

Surface	Purpose	# of Strains	Source/Food	Conclusion	Reference
Polyvinyl chloride	To compare abilities of <i>L.monocytogenes</i> strains to form biofilms	31	Culture collection	Biofilm production varied greatly between strains after 40 hours	Djordjevic et al., 2002.
Glass	Evaluate adhesion and biofilm growth	13	Human, meat and rabbit	After 3 hours strains attached to surface and at 24 hours all strains formed biofilms	Chae and Schraft, 2000.
Polystyrene, plastic and stainless steel	Assess biofilm-forming ability of <i>L.monocytogenes</i>	3	1985 listeriosis outbreak	All strains were able to consistently form biofilms using various methods	Marsh et al., 2003.
Stainless steel	Establish adherence to stainless steel at different temperatures and in multispecies	2	Culture collection	Both strains adhered at all temperatures with 18°C being optimum and both formed biofilms better in monoculture	Norwood and Gilmour, 2001.
Stainless steel and poyltetrafluoroethylene	To assess surface properties and biofilm growth	1	Culture collection	Biofilms formed on both surfaces	Chavant et al., 2002.
Stainless steel	Examine biofilm-forming capability	1	Outbreak, source unknown	Formed a biofilm in controlled conditions but not in model food system	Hood and Zottola, 1997.
17 materials representing metal, rubber and polymers	Determine adhesion to different materials	1	Culture collection	Adhesion to materials was time-dependent	Beresford et al., 2001.
Stainless steel	Ability to form a biofilm	36	Clinical, food and environment	Strains differed in ability to form a biofilm and most did not	Kalmokoff et al., 2001.

Table 3. Applications of PFGE to *Salmonella* typing

Application	Purpose	# of Strains	Source/Food	Conclusion	Reference
Tracking	Determine prevalent strains in broiler plant and source	79	Litter, feces, environmental swabs	Sources are company specific and some strains are persistent	Leibana et al., 2002.
Epidemiological Surveillance	Establish typing data for <i>Salmonella</i> due to increase in infections	55	Food-borne outbreak diarrhea	Most strains are recirculated and highly related	Tsen and Lin, 2001.
Outbreak Investigation	Type strains to aid in epidemiological investigation	10	Poultry	Source was poultry from local supplier and all strains are related	Moore et al., 2003.
Epidemiological Surveillance	Analyze and observe <i>Salmonella</i> patterns to determine if there is a geographical relationship	441	Clinical, food and poultry	Genetic diversity, replacement and expansion of Enteritidis subtypes associated with epidemic changes	Fernandez et al., 2003.
Outbreak Investigation	Type strains to see if there is a relationship	28	Eggs possible source	Single strain was the cause but source not determined	Murase et al., 1996
Epidemiological Surveillance	To establish an international database of DNA profiles	212	Humans, food and environment	Computerized analysis is helpful for long-term epidemiological comparison and surveillance	Garaizar et al., 2000.
Epidemiological Surveillance	To compare patterns from Denmark, Spain and England	101	Animal and human	All strains showed a close genetic relationship	Laconcha et al., 2000.
Tracking	Establish molecular markers to determine common origin	291	Poultry-related	Increased risk of infection was associated with the feed mills	Chadfield et al., 2001.
Epidemiological Surveillance	To determine significance of chicken meat as vehicle for human infection	53	Human, chicken meat, chicken feces	45 out of 53 had indistinguishable patterns and other had similar patterns, related to a spread of identical clone	Boonmar et al., 1998.
Tracking	Investigate chicken and egg facilities for <i>Salmonella</i> contamination	257	Chicken, eggs	Contamination originated in chicken house and egg belts are likely source of spreading <i>Salmonella</i> from 1 house to the others	Murase et al., 2001.

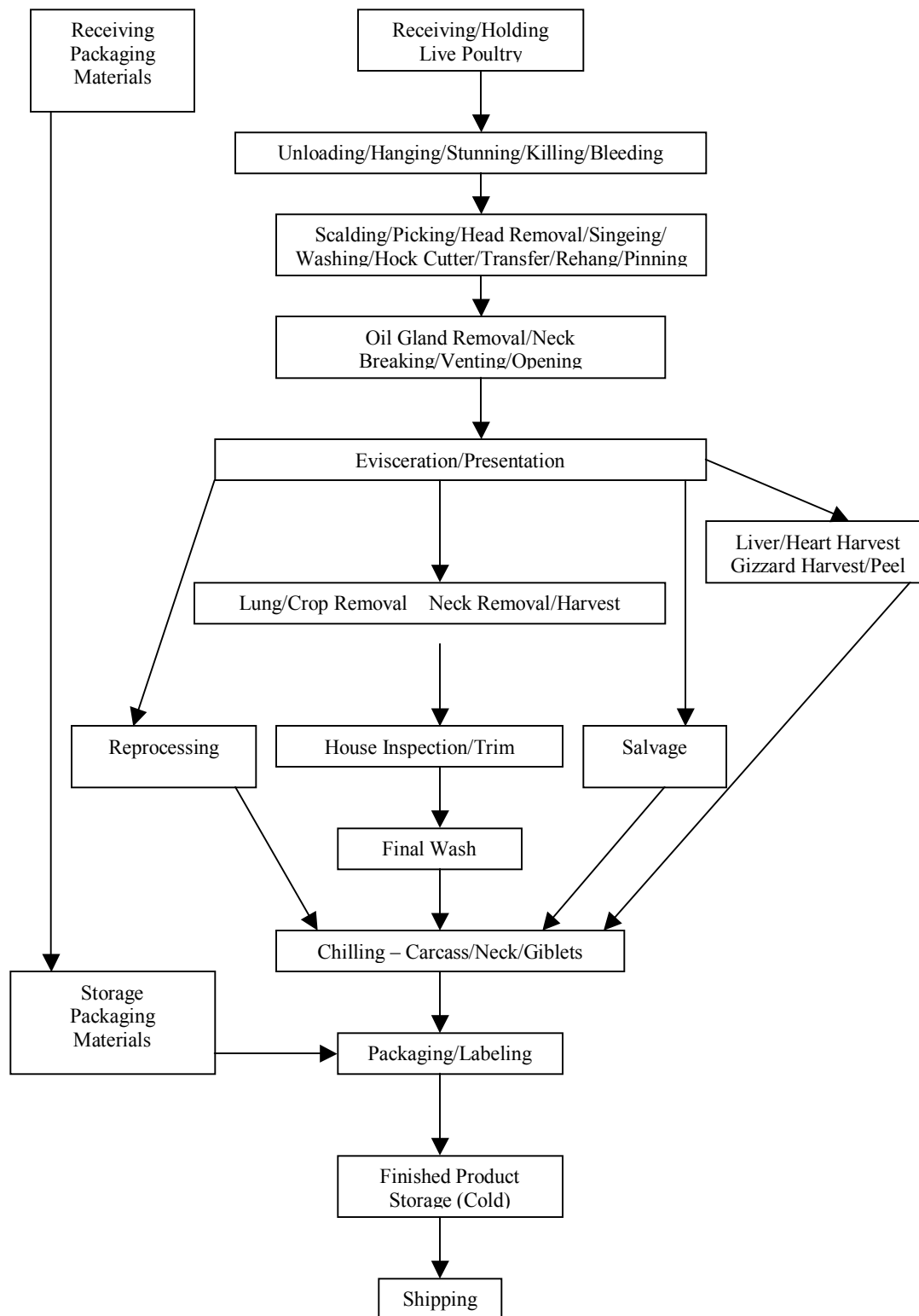


Figure 1. Processing flow chart for Poultry processing facility

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<http://www.ers.usda.gov/Briefing/FoodborneDisease/features.htm>

<http://www.ers.usda.gov/Briefing/FoodborneDisease/foodandpathogens/>

CHAPTER 2

Prevalence and Persistence of Select Foodborne Pathogens in a Mid-Atlantic Turkey

Processing Facility

Abstract

Listeria monocytogenes, *Salmonella* and *Campylobacter* combined are responsible for the majority of foodborne disease hospitalizations and over 1200 deaths annually in the U.S. alone. Although raw poultry has been identified as a source of these pathogens, most microbiological studies have focused on broilers with little attention given to turkey processing. The purpose of this research was to investigate the prevalence of select pathogens (*L. monocytogenes*, *Salmonella* spp., and *Campylobacter* spp.) and microbiological indicators (*Enterococcus* spp.) in the turkey processing plant environment. Environmental samples were collected in one Southeastern processing facility using swab methods at two month intervals over a period of 14 months. Samples were taken from conveyors, drains, walls and various food contact surfaces. Isolation and identification of bacteria was done using the USDA-FSIS Microbiology Laboratory Guidebook protocols. The prevalence of contamination was 11.5%, 7.4%, and 0.4% for *L. monocytogenes*, *Salmonella*, and *Campylobacter*, respectively. *Enterococcus* spp., an environmental indicator of fecal contamination, were isolated from over >75% of the samples screened. *Salmonella* isolates were typed using pulsed-field gel electrophoresis (PFGE) and *Enterococcus* isolates were speciated by PCR with antibiotic resistance profiles characterized using the SensiTitre system. A diverse set of relatively non-persistent *Salmonella* strains were obtained from the processing environment, as evaluated by PFGE. Thirty-nine percent of the *Enterococcus* isolates were speciated as

E. faecium and 55% were *E. faecalis*. Both *E. faecalis* as *E. faecium* strains were susceptible to most antibiotics of human clinical relevance. Thirty-three *L. monocytogenes* strains were screened for their biofilm formation capabilities using a microtiter well assay. None of the strains formed a biofilm in monoculture; however, sixteen of the strains were able to form a biofilm in the presence of another organism. Data collected in prevalence studies such as this one can help processors identify contamination frequency and sites in an effort to control resident pathogenic bacteria in the processing environment.

Introduction

It is estimated that 76 million illnesses a year are caused by foodborne pathogens (Mead et al., 1999). Of those, 14 million illnesses occur from known pathogens (Mead et al., 1999), with the bacterial pathogens of greatest concern being *Listeria monocytogenes*, *Campylobacter* spp. and *Salmonella* (<http://www.ers.usda.gov/Briefing/FoodborneDisease/foodandpathogens/>). *Listeria monocytogenes* and *Salmonella* account for approximately 80% of all deaths from known foodborne disease agents (Mead et al., 1999). In 1999 alone, all three microorganisms combined were responsible for most of the foodborne disease hospitalizations and over 1200 deaths (Mead et al., 1999). The U.S. economic impact of illness associated with *L. monocytogenes*, *Campylobacter* and *Salmonella* has been estimated at 5.9 billion a year (<http://www.ers.usda.gov/Briefing/FoodborneDisease/features/>). Interestingly, since the onset of the CDC FoodNet program, the incidence of listeriosis and campylobacteriosis appear to be steadily decreasing, while the incidence of *Salmonella* infection has continued to increase over the 3 year period from 2000 to 2002 (Anonymous, 2003; Anonymous, 2002; Anonymous, 2001).

Raw poultry has been identified as a source for these three important foodborne pathogens (Uyttendaele et al., 1999). In fact, 10 to 18.5% of all reported foodborne disease outbreaks are associated with poultry, with turkey responsible for 56% and chickens responsible for 44% of these (Bryan and Doyle, 1995; Coleman et al., 2003). In turkey carcasses, *Campylobacter* is usually present in 90% and *Salmonella* in 2.6% (Zhao et al., 2001; Lam et al., 1992). Approximately 1.2% of turkeys and 2.2% of broiler flocks are contaminated with both *C. jejuni/coli* and *Salmonella* (Harns et al., 1986;

Wedderkipp et al., 2001). Unfortunately, most microbiological studies aimed at estimating the incidence of foodborne pathogen contamination in poultry have focused on broilers, with relatively less attention given to turkey processing.

Enterococcus spp. have become a concern in the United States due to their ubiquitous nature and recent clinical disease associated with this genus. In particular, multiple-drug-resistant *E. faecalis* and *E. faecium* are the third leading cause of all nosocomial infections in intensive care units (Johnston and Jaykus, 2004; Eaton and Gasson, 2002; Hayes et al., 2004). Historically, *E. faecalis* has been the dominant causative agent of these infections, but a recent increase in *E. faecium* infections is believed to be due to the emergence of vancomycin-resistance enterococci (VRE) in food animal production (Eaton and Gasson, 2002; Hayes et al., 2004). In the European Union, VRE have been found in broilers and pigs, and has been linked to the use of a human antimicrobial glycopeptide, avoparcin, as a growth promoter in animal production (Manson et al., 2004; Hayes et al., 2004). Avoparcin use in animal production is thought to be responsible primarily for nonhospitalized increases in VRE infections in the European Union (Hayes et al., 2004).

Pathogenic and spoilage bacteria have been shown to attach to a wide variety of food-contact and non-contact surfaces (Lindsay et al., 1996; LeeWong, 1998). Such attachment of microorganisms to food contact surfaces, frequently referred to as biofilms, can impact the food industry by complicating sanitation and increasing the risk of cross contamination with spoilage and pathogenic microorganisms (Austin et al., 1998). *E. coli* O157:H7, *Salmonella enterica* serovar. Enteritidis, *L. monocytogenes*, and *C. jejuni* have been found to adhere to a variety of surfaces and have been resistant to sanitizers

(LeeWong, 1998; Trachoo et al., 2002; Beresford et al., 2001; Joseph et al., 2001; Bonafonte et al., 2000). Furthermore, many different food contact surfaces are prone to biofilm production, including glass, rubber, plastics, aluminum, stainless steel, Teflon, Buna-N (dairy processing) and rubber “fingers” (poultry processing). Floor drains and crevices in floors, as well as dead ends, joints, valves and gaskets of processing equipment, are also at risk for biofilm development (LeeWong, 1998).

The strains of pathogenic bacteria that colonize turkey processing plants and contaminate turkey products have not been characterized. There is a clear need to further investigate prevalence, strain subtypes and key strain attributes of these pathogens in this processing environment. This study was part of a broader project, the purpose of which was to investigate prevalence of select pathogens in the turkey processing industry by a systematic examination of environmental and product-associated contamination in processing facilities distributed in three geographical regions of the country (Eastern Seaboard, Midwest, West Coast). Accordingly, in this project, we report on the prevalence of *Listeria monocytogenes*, *Campylobacter*, and *Salmonella* spp. in a single processing facility located in the mid-Atlantic States. We also had the opportunity to isolate *Enterococcus* spp. also naturally present in this environment, and characterize their antibiotic resistance profiles. *Salmonella* isolates were serotyped and genotyped, while *L.monocytogenes* strains were characterized for their ability to form biofilms. Finally, in cooperation with a multi-national media manufacturer, we had the opportunity to evaluate the performance of a new commercial *Listeria* selective medium as applied to *Listeria* isolation from our environmental sample enrichments.

Materials and Methods

Sampling Procedure

All samples were collected from a mid-Atlantic turkey processing plant every other month for 14 months. Samples were collected only during the first shift while lines were operating and during sanitation breaks. All environmental samples were taken of a 3 x 3in area by wiping the surface with the sterile sponge in a back and forth motion according to the protocol established by International BioProducts Inc. [SpongeSicle, International BioProducts Inc., Bothell, WA]. Swabs were placed in a cooler for transport back to the laboratory. Samples were processed the same day for *Salmonella*, *Campylobacter*, *Listeria monocytogenes* and *Enterococcus*. Sponge swabs were aseptically cut into 4 equal pieces using scissors. Unless otherwise specified all microbiological methods followed the protocol in the USDA-FSIS Microbiology Laboratory Guidebook (2002).

Isolation of *Listeria monocytogenes*

The swab was resuspended in 50ml of modified university of vermont broth (Becton Dickinson and Co., Sparks, MD) and incubated at 30°C. After 24 hrs 0.1ml of the sample was transferred to Fraiser broth (Becton Dickinson and Co.) and incubated at 37°C. Twenty-four hours later a *Listeria* Visual Immunoassay (Tecra International Pty Ltd., Australia) kit was performed according to the manufacturers' instructions. Two-hundred microliters of fraiser broth was transferred into a sterile glass test tube and placed in a boiling water bath for 15 minutes. The heated sample was transferred into individual wells in 200µl aliquots. The wells were covered with glad cling wrap and

incubated for 30min at 37°C. Wells were emptied by quickly inverting the contents into a waste container. Residual liquid was removed by striking the holder several times face down onto absorbent paper towels. Using a nozzle squeeze bottle, the wells were washed with the supplied Tecra Wash Solution 3 times. 200µl of conjugate solution supplied in the kit was added to each well. The wells were covered with cling wrap and incubated for 30min at 37°C. After the 30min, the wells were washed 4 times with the supplied Tecra Wash Solution. 200µl of substrate was added to each well and incubated at room temperature for 10min. Test results were read using the supplied Tecra Color Card to verify proper color change. All presumptive positive samples were streaked onto modified oxford agar (Becton Dickinson and Co.) and incubated for 24 hrs at 37°C. Suspect colonies, approximately 5 were transferred to 5% sheep blood agar (Remel, Lenexa, KS) and incubated at 37°C for 24 hrs. β-lysin CAMP factor test was performed on suspect colonies by placing a β-lysin disc (Remel) in the center of a 5% sheep blood agar plate, and streaking 4-8 isolates in straight lines away from the disc. Plates were incubated at 37°C for 35hrs. Positive samples were determined by an arrowhead shaped β-hemolysis zone around the disc. Presumptive *Listeria* Samples were verified using a MicroID test kit (Remel).

Isolation of *Salmonella*

The swab was enriched in 50ml of Buffered Peptone Water and incubated at 37°C. After 24 hrs 1ml of the sample was transferred to Tetrathionate broth (Becton Dickinson and Co.) and 0.1ml was transferred to Rappaport Vassiliadis broth (Oxoid Ltd, Hampshire, England) and incubated for 20 hrs at 42°C. One ml of the Tetrathionate and Rappaport Vassiliadis broths were transferred to individual pre-warmed M-broth tubes

(Becton Dickinson and Co.) and incubated at 37°C. After 24 hrs a *Salmonella* VIA (Tecra International Pty Ltd) kit was run according to the manufacturers' instructions and as described above. Presumptive positive samples were streaked on double modified lysine iron agar (Oxoid Ltd), xylose lysine tergitol 4 and brilliant green sulfa agar (Becton Dickinson and Co.) and incubated at 37°C for 48 hrs. Select colonies, approximately 5 were inoculated on to triple sugar iron and lysine iron slants (Becton Dickinson and Co.) and incubated for 24 hrs at 37°C. All presumptive positive samples were sent to the Ames, IA USDA APHIS National Veterinary services laboratories for further serotyping.

Isolation of *Campylobacter*

The swab was enriched in 50mL Hunt Enrichment Broth (Oxoid; Becton Dickinson and Co.; Sigma, St. Louis, MO) in a quart-size ziplock freezer bag (S.C. Johnson and Son, Inc., Racine, WI), gased with a mixture of 5% O₂, 10% CO₂ and 85% N₂ and incubated at 37°C for 4 hrs. The enrichment was then re-gased and incubated for an additional 20 hrs at 42°C. Samples were then streaked onto modified *Campylobacter* charcoal differential agar (Oxoid) and incubated in an anaerobic jar using CampyPak (Remel) for 24 hrs at 42°C. Presumptive colonies were inoculated in Brucella-FBP (Becton Dickinson and Co.) tubes and incubated in an anaerobic jar for 48 hrs at 42°C. Using the Brucella-FBP broth, semisolid brucella glucose medium (Becton Dickinson and Co., Sigma) was inoculated and incubated at 42°C for 1-3 days. A positive reaction will produce a yellow color; since *Campylobacter* is non-fermentative, the color of the medium will remain red-orange. Antibiotic resistance testing was carried out by spread plating 6 drops of BFBP broth (Becton Dickinson and Co.) onto Brucella-FBP agar

(Becton Dickinson and Co.). A disc of 30µg nalidixic acid (Becton Dickinson and Co.) and a disc of 30µg cephalothin (Becton Dickinson and Co.) were aseptically placed on the Brucella-FBP agar using a single disc dispenser (Becton Dickinson and Co.). Plates were incubated in an anaerobic jar at 42° C for 1-3 days. Antibiotic resistance was determined by a clear zone around the discs.

Isolation of *Enterococcus*

The procedure for the isolation of *Enterococcus* was done according to U.S. Food and Drug Administration (Simjee et al., 2002; Hayes et al., 2003; D. D. Wagner [Food and Drug Administration], personal communication). Samples were first enriched in enterococcosel broth (Becton Dickinson and Co.) at 44°C for 48 hours and then streaked on enterococcosel agar (Becton Dickinson and Co.) and incubated at 37°C. After 24 hours, colonies showing esculin hydrolysis were streaked on Brain Heart Infusion agar (Becton Dickinson and Co.) and incubated at 37°C for 24 hours. The samples were then inoculated into BHI broth (Becton Dickinson and Co.) and incubated at 37°C. After 24 hours, samples were frozen in a 40% glycerol (Sigma) stock with brain heart infusion (Becton Dickinson and Co.) for further analysis by PCR.

PCR of *Enterococcus* genus

For PCR, DNA was extracted with the Ultra Clean microbial DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA) in accordance with manufacturer recommendations. Primers were directed to the *tuf* gene (forward primer, TACTGACAACCATTCATGATG; reverse primer, AACTTCGTCACCAACGCGAAC), yielding a 112-bp product. Five microliters of

DNA was added to a 45µl mixture containing 100nM Tris pH 8.3, 25mM MgCl₂, 10mM dNTP's, 0.3µl concentration of each primer, 29.4µl of DNase free water and 1µl of *Ampli-Taq* polymerase. The samples were subjected to an initial denaturation at 95°C for 3 min in the GeneAmp PCR System 9600 (Perkin Elmer, Wellesley, MA), followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 70 sec, and a final cycle of 72°C for 7 min and a cool down to 4°C. Isolates producing an amplicon band of the appropriate size by agarose gel (1%) electrophoresis were considered presumptively positive for the genus *Enterococcus* and were further tested with API test strips for species-level identification.

PCR of *Enterococcus* species

For *E. faecalis* identification primers were directed to the *ddl* gene (forward primer, ATCAAGTACAGTTAGTCT; reverse primer, ACGATTCAAAGCTAACTG), yielding a 941-bp product. Five microliters of DNA was added to a 45µl mixture containing 10mM Tris pH 8.3, 2.5mM MgCl₂, 10mM dNTP's, 0.3µl concentration of each primer, 29.4µl of DNase free water and 1µl of *Ampli-Taq* polymerase. The samples were subjected to an initial denaturation at 94°C for 2 min in the GeneAmp PCR System 9600 (Perkin Elmer, Wellesley, MA), followed by 30 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, and a final cycle of 72°C for 10 min and a cool down to 4°C. Isolates producing an amplicon band of the appropriate size by agarose gel (1%) electrophoresis were considered presumptively positive for the *E. faecalis* species.

For *E. faecium* identification the primers used were the forward primer, TTGAGGCAGACCAGATTGACG; reverse primer, TATGACAGCGACTCCGATTCC, yielding a 658-bp product. Five microliters of DNA was added to a 45µl mixture

containing 10mM Tris pH 8.3, 3.5mM MgCl₂, 200μM dNTP's, 1.0μM concentration of each primer, 29.4μl of DNase free water and 1μl of *Ampli-Taq* polymerase. The samples were subjected to an initial denaturation at 94°C for 4 min in the GeneAmp PCR System 9600 (Perkin Elmer, Wellesley, MA), followed by 25 cycles at 94°C for 30 sec and 72°C for 1 min, and a final cycle of 72°C for 8 min and a cool down to 4°C. Isolates producing an amplicon band of the appropriate size by agarose gel (1%) electrophoresis were considered presumptively positive for the *E. faecium* species.

Enterococcus API Test Strips

Species-level identification of *Enterococcus* isolates was done using the API 20 Strep test kits (BioMerieux, Hazelwood, MO). *Enterococcus* isolates were grown overnight in brain heart infusion (Becton Dickinson and Co.) at 37°C. Samples were streaked on to Columbia Blood Agar (Remel) and incubated at 37°C for 24 hrs. Colonies were harvested from the subculture plate and transferred into 2ml of distilled water, yielding a suspension turbidity of greater than 4 McFarland. Test strips were prepared and inoculated according to the manufacturers' instructions and incubated at 37°C for 24 hrs. After 24 hrs, reagents were added to the wells as specified in the manufacturers' instructions and results read after 10 mins.

Antibiotic Resistance Testing of *Enterococcus*

Strains identified as *E. faecium* or *E. faecalis* were screened for antibiotic susceptibility by the microdilution broth method with Mueller-Hinton media (TREK Diagnostics, Westlake, OH) as outlined by the National Committee on Clinical Laboratory Standards (NCCLS). A customized panel of 17 antibiotics with various

concentration ranges (TREK Diagnostics), identical to that used in the National Antimicrobial Resistance Monitoring System (NARMS 2001) program for gram-positive organisms was used in this study. The antibiotics and their concentration ranges were as follows: bacitracin, 8 to 128 IU/ml; chloramphenicol, 2 to 32 µg/ml; erythromycin, 0.5 to 8µg/ml; bambamycin (flavomycin), salinomycin, vancomycin, quinupristin-dalfopristin, and lincomycin, 1 to 32 µg/ml; penicillin, 0.5 to 16µg/ml; tetracycline, 4 to 32µg/ml; tylosin tartrate, 0.25 µg/ml; ciprofloxacin, 0.12 to 4 µg/ml; linezolid, 0.5 to 8µg/ml; nitrofurantoin, 2 to 128 µg/ml; kanamycin and gentamicin, 128 to 1,028µg/ml; and streptomycin, 512 to 2,048µg/ml. MICs were determined manually by assessing each antibiotic strain combination for growth. Isolates were categorized as susceptible, intermediate, or resistant, based on the NCCLS interpretive standards. The MICs, based on NCCLS breakpoints, were as follows: chloramphenicol and vancomycin, $\geq 32\mu\text{g/ml}$; erythromycin and linezolid, $\geq 8\mu\text{g/ml}$; penicillin and tetracycline, $\geq 16\mu\text{g/ml}$; quinupristin-dalfopristin and ciprofloxacin, $\geq 4\mu\text{g/ml}$; nitrofurantoin, $\geq 128\mu\text{g/ml}$; gentamicin, $>500\mu\text{g/ml}$; and streptomycin, $>1,000\mu\text{g/ml}$.

Long Term Storage of Cultures

All positive cultures were grown at 37°C overnight in brain heart infusion broth (Becton Dickinson and Co.). 500µl of the culture was pipetted into 1.0ml Nunc cryotube (Nalge Nunc International Corp., Rochester, NY) containing 500µl of a 40% glycerol (Sigma) stock in brain heart infusion broth. CryoTubes were stored at -20°C indefinitely.

CHROMagar *Listeria*

Isolation of *Listeria monocytogenes* was done according to the previous described protocol up to the incubation of the fraiser broth (Becton Dickinson and Co.). A loop full

of fraiser broth (Becton Dickinson and Co.) was streaked onto the CHROMagar *Listeria* plates (Becton Dickinson and Co.). Plates were incubated at 37°C for 24 hrs, examined and reincubated at 37°C for another 24hrs. Blue colonies having a halo around the colony were considered presumptive positives according to the manufacturers' instructions. Colonies showing a halo were then streaked onto 5% sheep blood agar (Remel) and incubated for 24 hrs at 37°C. Plates were then checked for β -hemolysis and then subjected to the previously summarized procedures.

Biofilms

The microtiter plate assay was done in accordance with the method reported by Djordjevic et al. (2002). *Listeria monocytogenes* strains were grown at 32°C for 24 hrs in 10ml of tryptic soy broth with 0.6% yeast extract (Becton Dickinson and Co.). Cultures, in 0.1ml aliquots, were transferred to 10ml of Modified Welshimer's Broth (Sigma or Premaratne et al., 1991) and vortexed; 100 μ l aliquots of the inoculated broth are transferred into each of 8 wells of a polyvinyl chloride, 96 well, microtiter plate (Becton Dickinson and Co.) that had been pre-rinsed with 70% ethanol and air dried, per strain. Plates were made in duplicate, covered and incubated at 32°C for 20 and 40 hrs. Each plate included 8 wells of Modified Welshimer's Broth without culture as controls. One hundred microliter aliquots of *L. monocytogenes* strain, *P. aeruginosa* ATCC 23993, *P. fluorescens* ATCC 13525, *P. fragi* ATCC 4973, *S. aureus* ATCC 25923 and *S. xylosus* ATCC 2997 in MWB broth were individually inoculated into 8 wells, to measure biofilm growth of each strain in a monoculture. In addition, 50 μ l aliquots of the *L. monocytogenes* strain was inoculated into 8 wells of 5 rows. The other strains of *P. aeruginosa*, *P. fluorescens*, *P. fragi*, *S. aureus* and *S. xylosus* were each individually

added into a single row in 50µl aliquots to the 50µl of *L. monocytogenes* strain to create a mixed culture consisting of a total of 100µl. Cell turbidity was monitored and recorded after 20 and 40 hours of growth using a Sunrise Tecan microtiter plate reader (Crailsheim, Germany) at 595nm. Moreover, after 20 and 40 hrs, the medium was removed from the wells and each well was washed 5 times with double distilled water. Plates were air dried for 45 min and each well stained with 150µl of 1% crystal violet solution (DIFCO Laboratories, Detroit, MI). After staining, each well was again washed 5 times with double distilled water. Biofilms were visible as purple rings on the side of each well. Quantitative analysis was done by adding 200µl of 95% ethanol to destain the wells; 100µl from each well was then transferred to a new microtiter plate and the optical density of the crystal violet destaining solution measured at 595nm using the Sunrise microtiter plate unit. Statistical significance was determined from the difference from the control (MWB without inoculate) using the INSTAT program.

PFGE analysis

One ml of overnight cultures of *Salmonella* grown in Luria-Bertani Broth at 37°C (Becton Dickinson and Co., Sparks, MD) at were centrifuged at $13 \times 1000\text{m}^{-1}$ for 1 min. The pellets were twice washed in 0.1 M phosphate buffered saline (PBS) (Sigma, St. Louis, MO), pH 7.2 and resuspended in 0.8 ml PBS. Modifications to the procedure for nucleic acid extraction and restriction were carried out according to the described protocols (Liebana et al., 2002; Laconcha et al., 1998; Liesegang et al., 2002). Briefly, 25 µl of proteinase K (Qiagen, Valencia, CA) were added to 1 ml of washed cultures and mixed by gently inverting several times. Cells were mixed with 2% PFGE agarose (Bio-Rad, Hercules, CA) and 100µl was pipetted into gel plug molds and solidified at room

temperature. Plugs were then transferred into 50ml tubes containing 5ml of cell lysis buffer (Promega) and then incubated overnight at 54°C. Plugs were washed in 5ml of ultra pure water (Hydro, Research Triangle Park, NC) at 54°C for 15 minutes, and then rewashed 3 times each for 15 minutes with 5ml of 1 X TE buffer. The plugs were then sized to 2mm in width and placed for 5 minutes in 1.5 ml Eppendorf tubes containing 200µl of room temperature 1 X pre-restriction buffer (Promega), transferred to Eppendorf tubes with 200µl *Xba*I (Promega) restriction buffer and incubated overnight at 37°C. A 1% PFGE agarose gel was made using 0.5 X TBE. Before casting the gels the restriction mixture containing the DNA agarose plugs was removed, replaced with 200µl of 0.5 X TBE and incubated for 5 minutes at room temperature. The plugs were then loaded into the wells and sealed using 1% PFGE agarose in 0.5 X TBE. DNA fragments were separated using the contour-clamped homogeneous electric field method (CHEF-DR III System, Bio-Rad Laboratories). The 0.5 X TBE buffer was kept at a temperature of 14°C at 6 V/cm, with pulse times of 6 s and 40 s for 20 hours with an included angle of 120. Lambda DNA concatemers (New England Biolabs Inc., Beverly, MA) were used as molecular size standards. The gel was stained using an ethidium bromide solution with shaking for 20 min. and then destained in distilled water with shaking for 1 hr. Gel images were analyzed using an imager (Bio-Rad). The banding patterns were analyzed using BioNumerics (Applied Maths, Austin, TX)

Results

Prevalence of *Campylobacter*, *Salmonella*, and *Listeria monocytogenes* in the processing environment

Figure 2 shows the pathogen prevalence during the time period from September 2003 – October 2004. *Campylobacter* was isolated only once from the environmental samples, that isolation occurring in February, 2004. *Listeria monocytogenes* was isolated consistently throughout the entire sampling period except for August, 2004; the highest isolation occurred in June 2004, where 6/31 (19.4%) of samples were positive for *L. monocytogenes*. Likewise, *Salmonella* was frequently isolated from the turkey-processing environment. September of 2003 produced the highest *Salmonella* isolation rates, with a total of 9/40 (22.5%) positive samples. In general, *Salmonella* tended to be isolated from food contact surfaces which included tables and bins early in the processing timetable, i.e., during live hanging, evisceration, and defeathering (Table 4). *Listeria* spp. and *Listeria monocytogenes* were predominantly isolated from non-food contact surfaces, especially floors, catwalks and drains.

Prevalence of *E. faecalis* and *E. faecium* isolates in the processing environment

The distribution of *Enterococcus* samples in the processing environment, as determined for five sampling periods, is shown in Table 5. *Enterococcus* isolates were obtained in all areas of the processing environment at isolation rates ranging from 75-100%, and with no apparent trend with respect to sample type or location. Among the 211 isolates obtained from all of the samples, a total of 82 (39%) were *E. faecium*, 115 (55%) were *E. faecalis*, and 14 (7%) were other *Enterococcus* spp.

Antibiotic Resistance patterns of *E. faecalis* and *E. faecium*

In general, enterococci show intrinsic resistance to cephalosporins, lincosamides, and many synthetic β -lactams, such as the penicillinase-resistant penicillins (Giraffa, 2002; Prazak et al., 2002; Johnston and Jaykus, 2004). A summary of the resistance

profiles is provided in Table 6. *Enterococcus* species are also resistant to low levels of aminoglycosides due to the decreased uptake of this antibiotic class (Giraffa, 2002; Johnston and Jaykus, 2004). Our results are consistent with previous reports on inherent resistance of *Enterococcus* spp. For instance, both *E. faecium* and *faecalis* had intrinsic resistance to bacitracin, i.e. 90% of the isolates were inhibited at concentrations greater than 128 IU/ml. *E. faecium* also had a MIC₉₀ of greater than 32 µg/ml for flavomycin. Consistent with reported data (Hayes et al., 2003; Sengeløv et al., 2002; Johnston and Jaykus, 2004), a majority (98%) of *E. faecalis* isolates were resistant to quinupristin/dalfopristin. Virtually 100% of the *E. faecium* strains were also resistant to quinupristin/dalfopristin. Both *E. faecium* and *E. faecalis* strains were notably resistant to erythromycin (97% for *E. faecium* and 96% of *E. faecalis*) and tetracycline (85% for *E. faecium* and 97% for *E. faecalis*). Twenty-eight percent of *E. faecium* isolates were also resistant to penicillin. None of the isolates demonstrated resistance to vancomycin. Our results show only 1.2% vancomycin resistance for *E. faecium*, with 8% of the strains having intermediate resistance. Only 2% of the *E. faecalis* strains were resistant to vancomycin, and only in the intermediate range.

Evaluation of BD CHROMAgar on the detection of *L. monocytogenes*

Overall, the CHROMagar plating results matched those of the standard selective plating media used to isolate *Listeria*, producing no false positive or false negative results for either *Listeria* spp. or *L. monocytogenes* (Table 7). Also, there were only one or two presumptive *L. monocytogenes* isolates arising on any one CHROMagar plate, as compared to MOX plates, which were frequently loaded with presumptive positive

isolates for the *Listeria* genus. All colonies showing a halo on CHROMagar were also hemolytic on blood agar, and vice versa.

PFGE and *Salmonella* Serotype

A representative PFGE gel is shown in Figure 3, while the *Salmonella* serotype results are detailed in Table 8. During the first sampling period, 9 *Salmonella* strains were isolated, resulting in three different serotypes, i.e., *S. senftenberg*, *S. agona*, and *S. berta*. The PFGE results separate out by serotype, i.e., the four *S. agona* strains had an identical PFGE pattern, as did the four *S. berta* strains. The single *S. senftenberg* strain had a completely unique PFGE pattern. During the second sampling period, three *Salmonella* strains were obtained, and each had a unique PFGE pattern with two multiple serotypes being detected and *S. schwarzengrund*. Similar results were seen for the other sampling periods.

Biofilms

Thirty-three *L. monocytogenes* strains isolated from a turkey processing plant where evaluated along with strain Scott A and *L. innocua* as controls, for their potential to form biofilms. The *Listeria* strains were grown for 40 hours in a 96 well microtiter plate separately and also in combination with *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Staphylococcus aureus* and *Staphylococcus xylosus*. All single *Listeria* strains tested did not form a biofilm when grown in the absence of another organism. Seventeen of the strains, including Scott A, showed some potential for biofilm formation when in the presence of another organism, although there was substantial run-to-run variability with tests run in triplicate. Sixteen of the seventeen strains were able to form biofilms when grown with *S. xylosus*; of those 16 strains, 4 also formed a biofilm

with *S. aureus*. One strain was also able to produce a biofilm in the presence of *P. fragi* in addition to the two *Staphylococcus* strains. Only one *L. monocytogenes* strain was able to form a biofilm when combined with *P. aeruginosa* and *P. fluorescens* (Table 9).

Discussion

Pathogen Prevalence

L. monocytogenes has been an increasing concern to food processors because it is an opportunistic pathogen that is commonly found in the environment (Ojeniyi et al., 1996). Recent studies show that *L. monocytogenes* was isolated from 0.3% to 18.7% of samples collected from seven Danish poultry abattoirs (Ojeniyi et al., 1996), and about 20% of the samples collected from a poultry processing plant in France were also positive for *L. monocytogenes* (Chasseignaux et al., 2001; 2002). In some instances, the *L. monocytogenes* strains were environmentally persistent (Chasseignaux et al., 2001; Lawrence and Gilmour, 1995). In our study we found a total *L. monocytogenes* contamination rate of 11.5% over a 14 month period. The majority of the contamination was found post-slaughter, primarily on nonfood contact surfaces, at a rate of 35% of the samples taken. This is consistent with an incidence range of 31-62.5% for environmental samples reported in previous studies (Lawrence and Gilmour, 1995; Chasseignaux et al., 2002). Chasseignaux et al. (2002) found approximately a 28% contamination rate of *L. monocytogenes* from the processing equipment and in the poultry processing plant environment. This was also consistent with an 18% and 16% contamination rate found in two poultry processing environments (Chasseignaux et al., 2001). We were unable to find *L. monocytogenes* associated with the turkey carcasses tested in our study, but we were able to screen only four. Uyttendaele et al.(1997) found *L. monocytogenes* present

in 4.6-5.9% of turkey meat samples tested. This prevalence was lower than found on broilers, where Lawrence and Gilmour (1995) reported a 31% isolation rate. Chasseignaux et al. (2001) found a 21% contamination rate of poultry meat and poultry products.

In a study by Nayak et al (2004), 5% of environmental turkey production samples, taken from samples such as the caeca, litter, water, and feed, were contaminated with *Salmonella*. Jimenez et al. (2002) reported a 20% *Salmonella* contamination rate for broiler carcasses. In other studies, turkey meat samples had an 8.2% prevalence of *Salmonella*, with frequent types being *S. Enteritidis*, *S. Agona*, *S. Heidelberg* and *S. Senftenberg* (Nayak et al., 2003; Beli et al., 2001). Few studies exist which detail the isolation rate of *Salmonella* in environmental samples taken from poultry processing facilities. In our study, we reported a *Salmonella* isolation rate of 7.4%, which is clearly lower than the previously reported 20% contamination rate in broilers (Jimenez et al., 2002). The majority of *Salmonella* contamination was found during evisceration and prior to chilling at rates of 18.8%. This finding is similar to data reported by Jimenez et al. (2002) who found that 12.5-29% of broilers sampled at the chiller were positive for *Salmonella* (Jimenez et al., 2002). We saw that chilling yielded a decrease in *Salmonella*, with no carcass samples testing positive post-chill, although again, few carcass samples were tested (n = 4). Interestingly, other studies have reported an increase in the prevalence of *Salmonella* isolation after chilling of broilers (Jimenez et al., 2002; Sanchez et al., 2002).

In this study, *Campylobacter* was found at a prevalence rate of 0.4%, with 1/12 environmental samples (8%) positive, primarily from the live hanging location.

Although it is generally accepted that raw poultry carcasses are contaminated with *Campylobacter* at high rates, isolation rates have ranged from as low as 30-40% (Logue et al., 2003) to as high as 80-90% (Berndtson et al., 1992). Again, there are few studies documenting *Campylobacter* isolation rates from the poultry processing facility itself. It is generally recognized that rates of contamination on raw product decrease after chilling. For instance, other published data on poultry show significant levels of contamination prechill at 40-41%, with post chill levels dropping to 19-90% (Sanchez et al., 2002; Logue et al., 2003).

Enterococcus

Increased antimicrobial resistance has been observed in human bacterial pathogens, with *E. faecalis* and *E. faecium* being the greatest concern clinically because they are the third leading cause of nosocomial infections and are becoming increasingly more resistant (Hayes et al., 2004). Increases in vancomycin-resistant enterococci have been linked to the use of avoparcin in broilers (Manson et al., 2004). This practice is a concern for the transmission of vancomycin-resistant enterococci via the food chain and its impact on the efficacy of this drug and others in the clinical environment (Hayes et al., 2004).

The survey of antimicrobial resistance of *Enterococcus* isolates was based on NARMS protocol as applied to *E. faecalis* and *E. faecium* isolates obtained from the environment. The environmental samples consisted of drains, floors, walls and various food contact surfaces. *Enterococcus* spp. was isolated from over 75% of the samples. Of the isolates, 38% were identified as *E. faecium* and 54% as *E. faecalis*. These percentages are consistent with isolation rates obtained by Hayes et al (2004). *E. faecium*

and *E. faecalis* showed rates of resistance to erythromycin at 72% and 69% respectively. Resistance for both *E. faecium* and *E. faecalis* was higher than 80% for quinupristin-dalfopristin and tetracycline. These resistance rates are consistent with rates of resistance previously published (Hayes et al., 2004; Manson et al., 2004). However, Manson et al (2004) reported a 5.8% resistance rate of vancomycin for both *E. faecalis* and *E. faecium*. Our results show only a 0.5% vancomycin resistance rate for *E. faecium*.

Among the 17 antibiotics screened in this study, 7 are used in animal feed for growth promotion. These include bacitracin, flavomycin, penicillin, salinomycin, tetracycline, lincomycin, and tylosin (Johnston and Jaykus, 2004). Both *E. faecium* and *E. faecalis* demonstrated a high degree of susceptibility to each of these antibiotics except for penicillin. Thirty-five percent of *E. faecium* isolates more resistant to penicillin with only one percent of *E. faecalis* isolates resistant. Erythromycin is also used in livestock production, specifically for therapeutic purposes in chickens and turkeys (Johnston and Jaykus, 2004). In the case of our study, 97% of *E. faecium* isolates and 96% of *E. faecalis* isolates were resistant to erythromycin. Other antibiotics that have been used for growth promotion in poultry include, polymyxin B, efrotomysin, virginiamycin, penicillin, bacitracin, avoparcin and lincomycin (Feighner and Dashkevich, 1987). It should be clear that much of the *Enterococcus* resistance reported here was to antibiotics commonly used in animal agriculture.

All of the antibiotics used in the panel are of importance for human therapeutic use except for tylosin tartrate, salinomycin, and flavomycin. Penicillin, vancomycin, aminoglycosides, chloramphenicol, ciprofloxacin, and quinupristin-dalfopristin all have been used in the treatment of enterococcal infection either in combination therapy, for

optimal killing, or monotherapeutically. Synergistic treatment includes the use of an aminoglycoside with the addition of a cell wall-active agent, such as vancomycin or penicillin (Johnston and Jaykus, 2004). Of the antibiotics that are important for human therapeutic uses, both *E. faecium* and *E. faecalis* were highly susceptible to bacitracin, erythromycin, tetracycline, lincomycin, kanamycin, gentamicin, and streptomycin. Of the aminoglycosides, *E. faecalis* showed a 2% and *E. faecium* a 9.2% level of resistance to vancomycin, and for penicillin *E. faecalis* had a 1% resistance and *E. faecium* a 35% resistance. Overall, the degree of resistance to antibiotics of human clinical relevance was minimal.

Concern in the United States over the use of human antimicrobials in animal production has been growing, in large part because most antimicrobials in animal production are analogues of the antibiotics used to treat human infections, in particular the VRE (Hayes et al., 2004). However, the US has not been as aggressive as the European Union in banning the use of these antibiotics in animal production. To date, the actual importance of meat products, including poultry, to the evolution and transmission of VRE is unknown.

Biofilms

The microtiter plate assay was used as a simple and rapid method to assess the ability of the *L. monocytogenes* isolates to form biofilms. This method was chosen for its ease of use, and its consistency amongst replicates and between experiments, particularly when compared to microscopy (Djordjevic et al., 2002). These same investigators also used the microtiter plate assay to assess biofilm-formation capability of naturally occurring *L. monocytogenes* strains, finding strain-to-strain differences. We found the

microtiter biofilm assay to be quite variable, and there were considerable differences in results amongst replicates and between experiments. This is notable because some of the important control points (such as using the same lot of plates) were also controlled in our experiments. Because of this variability, we are only comfortable in concluding that certain strains have the potential to form biofilms. Therefore, additional studies will be needed to ascertain if any one strain is a consistent biofilm producer. Despite complaints of variability when using alternative methods, such additional studies should probably use glass or stainless steel surfaces and some sort of microscopic (epifluorescence, laser scanning, transmission electron microscopy) method to confirm that a biofilm has indeed been formed.

Our results, however, are consistent with other published studies that showed that various *L. monocytogenes* strains differed in their ability to form biofilms, and that most strains do not form biofilms under laboratory conditions (Kalmokoff et al., 2001). In studies conducted on the variability of different *L. monocytogenes* strains to form biofilms, it has been noted that differing lineages can have an impact on the strain's ability to form a biofilm. Specifically, *L. monocytogenes* has been broken into three lineages; Lineage I strains contain epidemic isolates from humans and animals, while Lineage II contains human and animal isolates, and Lineage III contains just animal isolates (Djordjevic et al., 2002). Lineage I isolates have been found to have a greater ability to form biofilms than those in Lineage II or III. Marsh et al.(2003) and Chae and Schraft (2000) also found in the biofilm forming capability variability of different *L. monocytogenes* strains. There has been speculation that some of the strains that did not form biofilms were simply slow growing, and that given enough time, they would grow

to levels that could result in biofilm formation. However, a 48 hour cutoff point was used in these previous studies (Djordjevic et al., 2002; Chae and Schraft, 2000; Marsh et al., 2003), and since we used a 40 hour cutoff, our studies are comparable. It may be that more than 40 hours are required for notable biofilm formation to occur, and evaluating this possibility would be a logical extension of the work reported here.

Microorganisms form biofilms to protect themselves against adverse conditions and can grow on a variety of surfaces from stainless steel to rubber, which are typically found in poultry processing plants. Both pathogenic and spoilage bacteria that have been isolated from poultry plants in the past (*Salmonella* spp., *Listeria* spp., *Staphylococcus* spp., and *Pseudomonas* spp.) have also been found to be biofilm formers (Lindsay et al., 1996; Møretrø et al., 2003). These biofilms can be comprised of single or mixed species (Kumar and Anand, 1998), although mixed species biofilms offer added protection because they are generally more stable and create a larger and thicker biofilm mass (Donlan, 2002). In one study, *L. monocytogenes* was found to grow only in the presence of an extracellular polymeric substance (EPS) producing microorganism such as *P. fragi* (Zottola and Sasahara, 1994). Yet in another study, *L. monocytogenes* adhered in greater numbers in a monoculture as compared to growth in the presence of *P. fragi* and *S. xylosus*, suggesting that the attachment by *L. monocytogenes* was compromised by the presence of other organisms (Norwood and Gilmour, 2001). For the strains that formed biofilms in our study, attachment of *L. monocytogenes* appeared to be enhanced when *S. xylosus* was also present, indicating better biofilm capability as compared to growth in monoculture.

Serotyping and PFGE

Salmonella serotyping has been done for many years, but is rapidly being replaced, or at least complemented, by molecular typing methods such as PFGE. Many of the studies that have used PFGE in further strain discrimination have focused on only one or two *Salmonella enterica* serovars (Amavisit et al., 2001; Hoszowski and Wasyl, 2001; Moore et al., 2003; Valdezate et al., 2000; Kumao et al., 2002; Lindqvist et al., 2002; Liu et al., 2002; Goh et al., 2002; Cormican et al., 2002). In a study by Tamada et al. (2001), investigators reported 25 different PFGE patterns with a 72% level of genetic similarity for various strains of *Salmonella enterica* serotype Typhimurium when using the *XbaI* restriction enzyme. To further differentiate *Salmonella* Enteritidis strains associated with an outbreak, Lukinmaa et al. (1999) was able to obtain different PFGE patterns when strains were digested simultaneously with both *SpeI* and *NotI*. The inclusion of two enzymes in the restriction digest improved the discriminatory ability of the PFGE. Our results confirm a solid relationship between serogroup and PFGE pattern. In fact, in most instances, the PFGE pattern was identical for strains of the same serotype that were collected during any one sampling period. This suggests that one or more *Salmonella* strains are resident in the processing facility at any one time, but such residence does not necessarily carry over for an extended period of time. This hypothesis is supported by serotype data, which also changes over time. Further conclusions regarding extended residence of strains in the processing environment can be made by dendrogram analysis of the PFGE data.

In comparison to other studies, Nayak et al. (2003), who serotyped a total of 69 isolates obtained from environmental samples in a turkey production facility, showed that *S. Heidelberg* was the most common serotype, accounting for 69% of the isolates. The

other serotypes included *S. Senftenberg* (10%), *S. Muenster* (10%), *S. Anatum* (3%) and *S. Worthington* (3%). In a study of turkey meat, Beli et al. (2001) isolated *S. Enteritidis* and *S. Agona* most frequently, both serotypes being implicated in turkey related salmonellosis outbreaks in humans. Other serotypes encountered in turkey meat include *S. Blockley*, *S. Reading* and *S. Saint Paul* (Beli et al., 2001).

A limited number of studies have sought to use molecular strain typing to track *Salmonella* in the poultry production and processing environment, or on carcasses or products intended for the retail market. In earlier studies, Boonmar et al. (1998) used phage typing and PFGE to demonstrate the spread of a genetically identical clone of *S. enteritidis* in humans and poultry in Thailand. Several studies have reported on the use of PFGE for epidemiological tracking and as a surveillance tool for investigating the persistence of *Salmonella* strains. In Canada, an outbreak due to *Salmonella enterica* serotype Enteritidis was traced back to the source of contamination using PFGE, however the clinical strains obtained could not be differentiated from non-outbreak strains (Ahmed et al., 2000). In an international study of *Salmonella enterica* serovar Bovismorbificans, a total of 28 PFGE patterns were identified, with 85% belonging to one PFGE type designation, indicating a clonal relationship between outbreak strains from Europe and Australia (Leisegang et al., 2002).

There are over 2300 serovars of *Salmonella* and only 50 of those are isolated from humans and animals (Uzzau et al., 2000). Of those serotypes isolated they belong to the subspecies *enterica* (Uzzau et al., 2000). *S. enterica* subspecies I are associated with poultry and mammalian disease (Baumler et al., 1998). The other subspecies classes II-VII are predominately associated with cold-blooded vertebrates (Baumler et al., 1998).

From the subspecies I, the types most frequently encountered in humans include the host-adapted Typhimurium, Enteritidis, Typhi, Sendai, Paratyphi A, B and C (Baumler et al., 1998). The subspecies encountered in poultry include host-adapted Pullorum and Gallinarum (Baumler et al., 1998; Uzzau et al., 2000).

Conclusions

The strains of pathogenic bacteria that colonize turkey processing plants and contaminate turkey products have not been characterized. There is a clear need to further investigate prevalence, strain subtypes and key strain attributes of these pathogens in this processing environment. This study was part of a broader project, the purpose of which was to investigate the prevalence of select pathogens in the turkey processing industry by a systematic examination of environmental and product-associated contamination in processing facilities distributed in three geographical regions of the country (Eastern Seaboard, Midwest, West Coast).

We can conclude based on the work presented here, which is based on the survey of a single processing facility in the mid-Atlantic U.S., that *L. monocytogenes* and *Salmonella* are present in the raw turkey processing environment at prevalence rates approximating 5-10%. *Enterococcus* spp. were isolated from over 75% of the environmental samples tested; antibiotic resistance profiles reflected both intrinsic resistance and resistance to antibiotics of relevance to animal agriculture, but not to those of relevance to human clinical medicine. A variety of *Salmonella* serotypes were present, and serotype corresponded with PFGE type. Of the *L. monocytogenes* strains isolated and characterized for biofilm capability, all were unable to form biofilms unless co-cultured with another microorganism. Even in mixed culture, most of the *L.*

monocytogenes strains still could not form biofilms, at least within the confines of the method chosen to evaluate biofilm production. Comprehensive studies such as these allow for the evaluation of microbial contamination in the real-world environment, and provide substantial data upon which future approaches to mitigate foodborne disease risk can be based

Table 4. Pathogen prevalence by processing areas

Processing Areas	<i>Campylobacter</i>	<i>Salmonella</i>	<i>L. monocytogenes</i>	<i>Listeria</i> spp.
Carcass	0/4	0/4	0/4	0/4
Drains	0/33	0/33	13/33 (39%)	4/33 (12%)
Conveyor	0/30	1/30 (3%)	0/30	0/30
Floors/Walls	0/10	0/10	5/10 (50%)	1/10 (10%)
Catwalk	0/17	0/17	3/17 (18%)	4/17 (24%)
Food Contact Surfaces				
Live-hanging	1/12 (8%)	3/12 (25%)	0/12	0/12
Defeathering	0/7	3/7 (43%)	0/7	2/7 (29%)
Eviseration	0/26	4/26 (15.4%)	0/26	0/26
Reprocessing	0/24	3/24 (12%)	1/24 (4%)	1/24 (4%)
Chiller	0/21	0/21	2/21 (10%)	1/21 (5%)
Rehang	0/5	0/5	1/5 (20%)	0/5
Osteo (cutting area)	0/7	1/7 (14%)	0/7	0/7
Roast (raw product packaging)	0/6	0/6	1/6 (17%)	0/6
Grinder	0/4	0/4	0/4	0/4
Other	0/38	3/38 (8%)	2/38 (5%)	1/38 (3%)

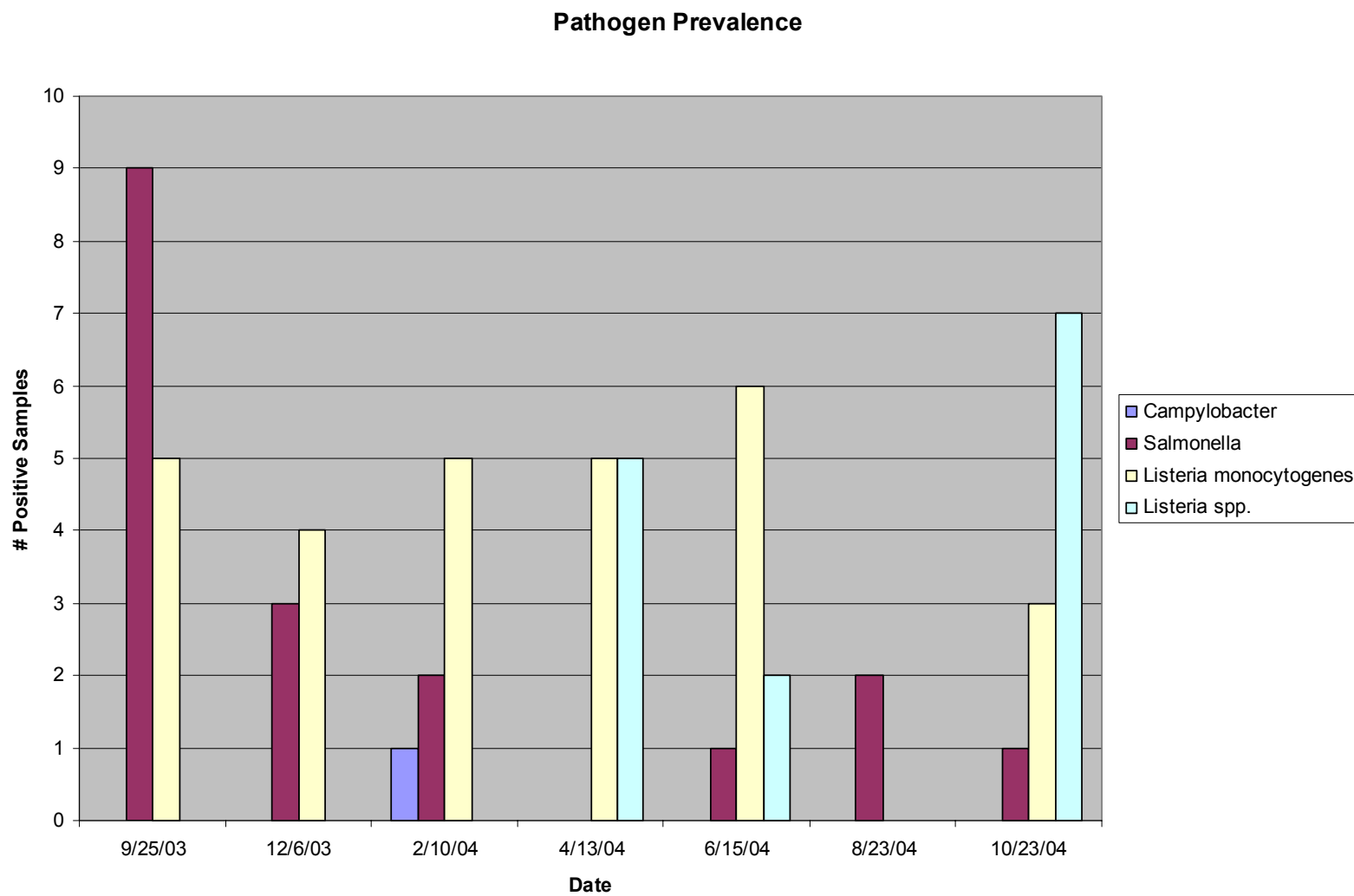


Figure 2. Prevalence of pathogens during various sampling times

Table 5. *Enterococcus* spp. environmental isolates

Processing Area (n = 186)	No. (%) of samples		No. (%) of <i>Enterococcus</i> isolates			
	Total n	With <i>Enterococcus</i> spp.	Total	<i>E. faecium</i> (n=82)	<i>E. faecalis</i> (n=115)	Other species
Carcass	2	2 (100)	2	1	1	0
Drains	26	24 (92)	31	9	17	5
Conveyor	23	23 (100)	26	8	17	1
Floors/Walls	5	5 (100)	7	0	5	1
Catwalk	14	14 (100)	16	7	9	0
Food Contact Surfaces						
Live-hanging	9	8 (89)	12	8	4	0
Defeathering	5	4 (80)	6	0	5	1
Evisceration	15	14 (93)	21	8	12	1
Reprocessing	19	19 (100)	20	9	11	0
Chiller	16	16 (100)	18	5	11	1
Rehang	4	4 (100)	4	1	3	0
Osteo	5	4 (80)	6	4	2	0
Roast	4	3 (75)	5	3	1	1
Grinder	2	2 (100)	2	1	0	0
Other	27	25 (93)	38	18	17	2

Table 6. Antibiotic resistance profiles of *E. faecium* and *E. faecalis*

Antibiotic	<i>E. faecium</i> (n=79)				<i>E. faecalis</i> (n=108)			
	Resistant (%)	Intermediate (%)	MIC ₅₀ ¹	MIC ₉₀ ²	Resistant (%)	Intermediate (%)	MIC ₅₀	MIC ₉₀
Bacitracin	NA ³	NA	>128	>128	NA	NA	>128	>128
Chloramphenicol	0	3 (3.7) ⁴	-	-	2 (2)	3 (3)	-	-
Erythromycin	57 (72)	20 (25)			74 (69)	29 (27)	-	-
Flavomycin	NA	NA	>32	>32	NA	NA	1	4
Penicillin	28 (35)	0	-	-	1 (1)	0	-	-
Salinomycin	NA	NA	1	4	NA	NA	1	4
Quinupristin/dalfopristin	68 (86)	11 (14)	-	-	99 (92)	7 (6)	-	-
Tetracycline	67 (85)	0	-	-	105 (97)	0	-	-
Vancomycin	1 (1.2)	6 (8)	-	-	0	2 (2)	-	-
Lincomycin	NA	NA	>32	>32	NA	NA	>32	>32
Tylosin Tartrate	NA	NA	>32	>32	NA	NA	>32	>32
Ciprofloxacin	1 (1.2)	17 (22)	-	-	0	2 (2)	-	-
Linezolid	1 (1.2)	0	-	-	2 (2)	0	-	-
Nitrofurantoin	1 (1.2)	10 (13)	-	-	0	1 (1)	-	-
Kanamycin	NA	NA	128	>1024	NA	NA	128	>1024
Gentamicin	NA	NA	128	>1024	NA	NA	128	>1024
Streptomycin	NA	NA	512	>2048	NA	NA	512	>2048

1. MIC₅₀ – 50% of isolates inhibited

2. MIC₉₀ – 90% of isolates inhibited

3. NA – Not applicable

4. Number without parenthesis indicates # of strains resistant or intermediately resistant to antibiotic; parenthetical results are percentages.

Table 7. Evaluation of BD ChromAgar

Date	Sample Code	MOX	BD	Blood Agar	Strain
April	148	+	growth, no halo	neg	NOT Lm
	154	+	growth, no halo	neg	NOT Lm
	158	+	(+), halo	+	Lm, not 4b
	171	+	(+), halo	+	Lm 4b
	172	+	growth, no halo	neg	NOT Lm
	174	+	growth, no halo	neg	NOT Lm
	176	+	(+), halo	+	Lm, not 4b
	178	+	growth, no halo	neg	NOT Lm
	179	+	(+), halo	+	Lm, not 4b
	180	+	(+), halo	+	Lm, not 4b
June	191	+	(+), halo	+	Lm, not 4b
	192	+	(+), halo	+	Lm, not 4b
	196	+	growth, no halo	neg	NOT Lm
	201	+	(+), halo	+	Lm, not 4b
	202	+	growth, no halo	neg	NOT Lm
	206	+	(+), halo	+	Lm, not 4b
	209	+	(+), halo	+	Lm, not 4b
	210	+	(+), halo	+	Lm, not 4b
October	257	+	growth, no halo	neg	NOT Lm
	265	+	(+), halo	+	Lm
	266	+	(+), halo	+	Lm
	268	+	(+), halo	+	Lm
	274	+	growth, no halo	neg	NOT Lm
	275	+	growth, no halo	neg	NOT Lm
	277	+	growth, no halo	neg	NOT Lm
	279	+	growth, no halo	neg	NOT Lm
	282	+	growth, no halo	neg	NOT Lm
	283	+	growth, no halo	neg	NOT Lm

Table 8. *Salmonella* Serotypes

Sample Date	Source	<i>Salmonella</i> serotype
9/25/2003	live hanging	<i>S. senftenberg</i>
9/25/2003	defeathering	<i>S. agona</i>
9/25/2003	handwashing sink	<i>S. agona</i>
9/25/2003	condemned bucket - evis area	<i>S. agona</i>
9/25/2003	conveyor from evis to chiller	<i>S. berta</i>
9/25/2003	reprocessing bucket	<i>S. berta</i>
9/25/2003	osteo bucket	<i>S. agona</i>
9/25/2003	neck table	<i>S. berta</i>
9/25/2003	gizzard table	<i>S. berta</i>
12/6/2003	live hanging	Multiple serotypes
12/6/2003	Defeathering	Multiple serotypes
12/6/2003	neck table	<i>S. schwarzengrund</i>
2/10/2004	evisceration trough	<i>S. reading</i>
2/10/2004	neck table	<i>S. senftenberg</i>
6/15/2005	evisceration bucket condemned	<i>S. derby</i>
8/23/2004	Boots	<i>S. muenchen</i>
8/23/2004	evisceration bucket condemned	<i>S. heidelberg</i>
10/23/2004	live hanging	<i>S. anatum</i>

Table 9. Biofilm Growth

<i>L.monocytogenes</i> strain	MWB	<i>Listeria</i>	<i>Listeria</i> + <i>P.</i> <i>aeruginosa</i>	<i>Listeria</i> + <i>P.fluorescens</i>	<i>Listeria</i> + <i>P.fragi</i>	<i>Listeria</i> + <i>S.aureus</i>	<i>Listeria</i> + <i>S.xylosus</i>
Scott A	0.25 ± 0.04 ¹	0.26 ± 0.07 ¹	0.45 ± 0.11 ¹	0.42 ± 0.08 ¹	0.46 ± 0.18 ¹	0.51 ± 0.24 ^{1*}	0.61 ± 0.37 ^{1*}
<i>L.innocua</i>	0.23 ± 0.03 ¹	0.26 ± 0.02 ¹	0.36 ± 0.09 ¹	0.33 ± 0.07 ¹	0.41 ± 0.22 ¹	0.39 ± 0.11 ¹	0.44 ± 0.16 ¹
11	0.17 ± 0.05 ¹	0.25 ± 0.03 ¹	0.35 ± 0.08 ^{1,2}	0.36 ± 0.10 ^{1,2}	0.39 ± 0.16 ^{1,2}	0.46 ± 0.14 ^{1,2}	0.65 ± 0.26 ^{2*}
18	0.13 ± 0.01 ¹	0.15 ± 0.01 ¹	0.31 ± 0.09 ¹	0.30 ± 0.11 ¹	0.35 ± 0.12 ¹	0.31 ± 0.19 ¹	0.46 ± 0.40 ^{1*}
22	0.16 ± 0.02 ¹	0.15 ± 0.01 ¹	0.26 ± 0.12 ¹	0.27 ± 0.15 ¹	0.30 ± 0.19 ¹	0.39 ± 0.28 ¹	0.50 ± 0.38 ^{1*}
24	0.12 ± 0.02 ¹	0.17 ± 0.04 ^{1,3}	0.22 ± 0.01 ^{2,3,4}	0.21 ± 0.01 ^{2,3,4}	0.21 ± 0.02 ^{2,3,4}	0.23 ± 0.03 ^{2,3,4}	0.28 ± 0.05 ^{2,4}
34	0.14 ± 0.03 ¹	0.16 ± 0.01 ^{1,3}	0.25 ± 0.04 ^{1,2,3}	0.21 ± 0.03 ^{1,2,3}	0.22 ± 0.04 ^{1,2,3}	0.27 ± 0.06 ^{2,3}	0.31 ± 0.06 ^{2,3}
43	0.12 ± 0.01 ¹	0.14 ± 0.03 ^{1,3}	0.21 ± 0.02 ^{2,4}	0.17 ± 0.02 ^{1,3,4,5}	0.18 ± 0.01 ^{2,3,4,5,6}	0.22 ± 0.02 ^{2,4,5,6}	0.27 ± 0.02 ^{2,4}
45	0.14 ± 0.02 ¹	0.20 ± 0.03 ¹	0.27 ± 0.03 ^{2,4}	0.20 ± 0.01 ^{1,3,5}	0.22 ± 0.02 ^{2,3,4,5,6}	0.25 ± 0.01 ^{2,3,4,5,6}	0.29 ± 0.04 ^{2,4}
52	0.16 ± 0.10 ¹	0.14 ± 0.03 ^{1,2}	0.22 ± 0.04 ^{1,2}	0.19 ± 0.02 ^{1,2}	0.26 ± 0.08 ^{1,2}	0.23 ± 0.04 ^{1,2}	0.32 ± 0.08 ¹
63	0.12 ± 0.01 ¹	0.13 ± 0.01 ¹	0.22 ± 0.05 ^{1,2}	0.20 ± 0.06 ^{1,2}	0.22 ± 0.08 ^{1,2}	0.28 ± 0.16 ^{1,2}	0.41 ± 0.25 ^{2*}
90	0.17 ± 0.09 ¹	0.19 ± 0.11 ¹	0.19 ± 0.01 ¹	0.20 ± 0.05 ¹	0.19 ± 0.07 ¹	0.20 ± 0.06 ¹	0.33 ± 0.07 ¹
102	0.11 ± 0.00 ¹	0.18 ± 0.03 ¹	0.29 ± 0.12 ¹	0.28 ± 0.14 ¹	0.36 ± 0.20 ¹	0.39 ± 0.23 ¹	0.56 ± 0.40 ^{1*}
104	0.12 ± 0.01 ¹	0.15 ± 0.01 ^{1,2}	0.24 ± 0.02 ^{1,2}	0.25 ± 0.02 ^{1,2}	0.25 ± 0.06 ^{1,2}	0.30 ± 0.14 ^{1,2}	0.37 ± 0.16 ²
105	0.11 ± 0.02 ¹	0.15 ± 0.01 ¹	0.26 ± 0.06 ^{1,2}	0.28 ± 0.10 ^{1,2}	0.32 ± 0.14 ^{1,2}	0.40 ± 0.20 ^{1,2}	0.62 ± 0.34 ^{2*}
128	0.13 ± 0.01 ¹	0.13 ± 0.01 ^{1,3}	0.20 ± 0.01 ^{2,4}	0.17 ± 0.01 ^{1,3,4,5}	0.19 ± 0.01 ^{2,4,5,6}	0.21 ± 0.02 ^{2,4,5,6}	0.30 ± 0.04 ²
138	0.14 ± 0.04 ¹	0.17 ± 0.01 ¹	0.28 ± 0.08 ¹	0.29 ± 0.10 ¹	0.31 ± 0.13 ¹	0.34 ± 0.20 ¹	0.54 ± 0.29 ^{1*}

Table 9 Biofilm growth (cont.)

<i>L.monocytogenes</i> strain	MWB	<i>Listeria</i>	<i>Listeria + P. aeruginosa</i>	<i>Listeria + P.fluorescens</i>	<i>Listeria + P.fragi</i>	<i>Listeria + S.aureus</i>	<i>Listeria + S.xylosus</i>
139	0.19 ± 0.07 ¹	0.23 ± 0.06 ¹	0.28 ± 0.04 ¹	0.26 ± 0.04 ¹	0.30 ± 0.07 ¹	0.34 ± 0.14 ¹	0.45 ± 0.27 ^{1*}
141	0.17 ± 0.04 ¹	0.20 ± 0.02 ¹	0.28 ± 0.05 ¹	0.26 ± 0.10 ¹	0.30 ± 0.15 ¹	0.31 ± 0.11 ¹	0.44 ± 0.20 ¹
143	0.14 ± 0.06 ¹	0.20 ± 0.06 ¹	0.32 ± 0.07 ¹	0.30 ± 0.11 ¹	0.33 ± 0.15 ¹	0.39 ± 0.19 ¹	0.57 ± 0.31 ^{1*}
158A	0.14 ± 0.05 ¹	0.19 ± 0.04 ^{1,3}	0.25 ± 0.05 ^{2,3}	0.19 ± 0.02 ^{1,3}	0.21 ± 0.03 ^{1,3}	0.21 ± 0.04 ^{1,3}	0.25 ± 0.02 ^{2,3}
158B	0.12 ± 0.02 ¹	0.18 ± 0.01 ^{1,3}	0.23 ± 0.01 ^{2,3}	0.19 ± 0.04 ^{1,3}	0.20 ± 0.03 ^{1,3}	0.21 ± 0.03 ^{1,3}	0.28 ± 0.06 ²
158C	0.21 ± 0.06 ¹	0.20 ± 0.01 ¹	0.38 ± 0.13 ¹	0.38 ± 0.15 ¹	0.49 ± 0.23 ¹	0.55 ± 0.26 ^{1*}	0.70 ± 0.37 ^{1*}
158D	0.14 ± 0.05 ¹	0.22 ± 0.07 ¹	0.36 ± 0.11 ¹	0.35 ± 0.17 ¹	0.43 ± 0.27 ^{1*}	0.51 ± 0.41 ^{1*}	0.61 ± 0.53 ^{1*}
171A	0.13 ± 0.01 ¹	0.16 ± 0.02 ¹	0.25 ± 0.04 ¹	0.22 ± 0.08 ¹	0.25 ± 0.13 ¹	0.29 ± 0.20 ¹	0.38 ± 0.30 ¹
171B	0.16 ± 0.01 ¹	0.17 ± 0.02 ¹	0.26 ± 0.03 ¹	0.23 ± 0.06 ¹	0.27 ± 0.11 ¹	0.32 ± 0.18 ¹	0.50 ± 0.27 ^{1*}
171C	0.18 ± 0.05 ¹	0.21 ± 0.04 ¹	0.23 ± 0.01 ¹	0.18 ± 0.01 ¹	0.20 ± 0.02 ¹	0.20 ± 0.01 ¹	0.25 ± 0.04 ¹
171D	0.13 ± 0.01 ¹	0.18 ± 0.01 ¹	0.27 ± 0.06 ¹	0.24 ± 0.09 ¹	0.28 ± 0.13 ¹	0.33 ± 0.21 ¹	0.47 ± 0.34 ^{1*}
176A	0.17 ± 0.06 ¹	0.26 ± 0.07 ¹	0.25 ± 0.04 ¹	0.20 ± 0.07 ¹	0.18 ± 0.02 ¹	0.20 ± 0.05 ¹	0.23 ± 0.03 ¹
176B	0.16 ± 0.04 ¹	0.18 ± 0.03 ¹	0.34 ± 0.05 ¹	0.32 ± 0.14 ¹	0.36 ± 0.18 ¹	0.41 ± 0.27 ¹	0.59 ± 0.44 ^{1*}
179	0.15 ± 0.04 ¹	0.19 ± 0.06 ^{1,3}	0.31 ± 0.06 ^{2,3}	0.24 ± 0.03 ^{1,3}	0.24 ± 0.05 ^{1,3}	0.25 ± 0.04 ^{1,3}	0.28 ± 0.03 ^{2,3}
180A	0.15 ± 0.05 ¹	0.20 ± 0.05 ¹	0.34 ± 0.13 ¹	0.30 ± 0.16 ¹	0.39 ± 0.22 ¹	0.45 ± 0.32 ^{1*}	0.60 ± 0.47 ^{1*}
180B	0.16 ± 0.06 ¹	0.19 ± 0.05 ^{1,3}	0.28 ± 0.06 ^{1,3}	0.20 ± 0.02 ^{1,3}	0.22 ± 0.02 ^{1,3}	0.24 ± 0.03 ^{1,3}	0.31 ± 0.06 ^{2,3}
180C	0.13 ± 0.05 ¹	0.19 ± 0.06 ^{1,3}	0.23 ± 0.05 ^{1,3}	0.19 ± 0.03 ^{1,3}	0.20 ± 0.03 ^{1,3}	0.24 ± 0.05 ^{1,3}	0.27 ± 0.03 ^{2,3}
180D	0.25 ± 0.10 ¹	0.23 ± 0.06 ¹	0.63 ± 0.41 ^{1*}	0.51 ± 0.22 ^{1*}	0.50 ± 0.17 ¹	0.48 ± 0.12 ¹	0.47 ± 0.19 ¹

*Strain is a potential biofilm former

1,2,3,4,5,6 Means that share the same superscript numbers are not significantly different from each other; means with different superscript numbers are significantly different (p < 0.05)

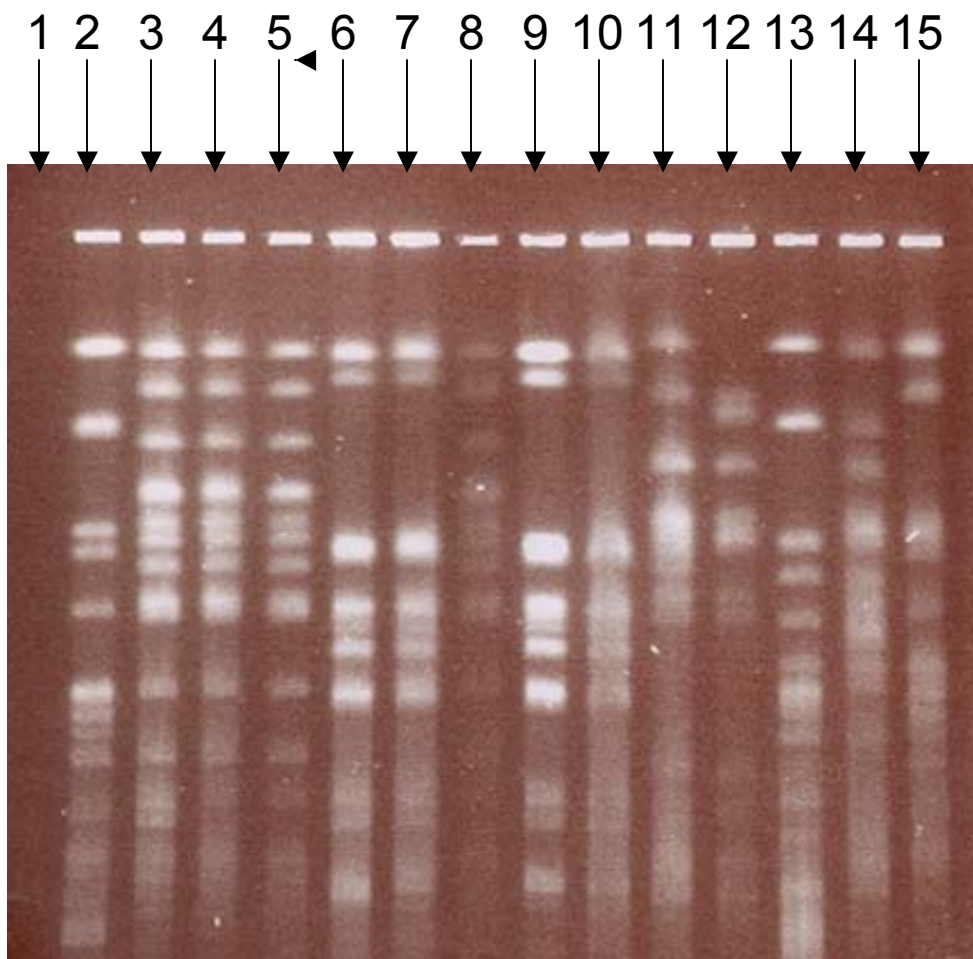


Figure 3. PFGE of *Salmonella*

1. Ladder
2. *S. Senftenberg*
3. *S. Agona*
4. *S. Agona*
5. *S. Agona*
6. *S. Berta*
7. *S. Berta*
8. *S. Agona*
9. *S. Berta*
10. *S. Berta*
11. Multiple serotype
12. Multiple serotype
13. *S. Schwarzengrund*
14. *S. Reading*
15. *S. Senftenberg*

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