5-2018

Microbiome of Commercial Broilers through Evisceration and Immersion Chilling

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Microbiome of Commercial Broilers Through Evisceration and Immersion Chilling

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

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May 2018
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ABSTRACT

The United States poultry industry generated 38.6 billion pounds (17,500 metric ton) of meat in 2014 which averaged to 121 pounds (55 kg) per individual of the U.S that same year. Of that meat generated by the poultry industry, an estimated 1 million cases of Salmonellosis will occur. Out of the 1 million cases approximately 40, 000 to 50,000 will be confirmed cases by the CDC. Recently, the USDA has requested changes in the inspection process and are currently allowing processors more freedom to utilize innovation to drive the increase in safer and more desirable foods. The new standards set forth by the USDA and the willingness to be more flexible with processors will create an atmosphere conducive for the development of new technologies, process design, and antimicrobial intervention strategies that are synergistic with the rate at which large scale production occurs. In this review, the production process will be explored in conjunction with the regulatory statutes that govern poultry slaughter. Additionally, the mechanism in which antimicrobials interact with bacteria and the employment of Next Generation Sequencing to gain better insight of how the intervention strategies decontaminate raw meat.

ACKNOWLEDGEMENTS

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Chapter 3: Handley JA, Park SH, Kim SA, and Ricke SC. Microbiome Profiles of Commercial Broilers Through Evisceration and Immersion Chilling During Poultry Slaughter and Identification of Potential Indicator Organisms. Front. Microbiol. doi:0.3389/fmicb.2018.00345

Chapter 1

Introduction

Raw meat products are susceptible to both human pathogens and spoilage organism that degrade the quality of sensory and shelf-life. The most recent census of foodborne illness associated with non-typhoidal *Salmonella* spp. estimates 1.0 million cases, 19,336 hospitalizations, and 378 deaths each year (Scallan *et. al*., 2011). As for spoilage or food waste, 39 billion pounds of meat, poultry, and fish harvested for retailers and consumers went un-eaten (Buzby *et. al.*, 2014). Foodborne illness and waste reports indicate the annual cost to be 51 billion and 48 billion US dollars (USD), respectively (Scallan *et. al*., 2011; Buzby *et. al*., 2014). In addition to foodborne illness and food waste, meat processors are subject to laws that govern the production of sanitary food destined for human consumption (FSIS-USDA, 1996b). These laws set forth rules in which processors are required to validate their production systems to prevent the contamination of meat products with human pathogens (FSIS-USDA, 1996a). When taking consideration to food borne illness, spoilage, and regulatory compliance, it is in the best interest of the meat industry to collaborate on matters pertaining to food safety.

Researchers and industry have worked together to address the industry's food safety needs by developing technologies, Good Manufacturing Practices, and Hazard Analysis Critical Control Point (HACCP) programs (Kramer *et. al.*, 2005). Throughout the evisceration process, there are many hurdles with antimicrobial properties, physical or chemical treatments, that reduce the microbial load of broilers brought to the slaughter facility. Birds are reportedly contaminated with aerobic bacterial levels between mean $\log_{10}$ 6 to 9 CFU/mL or 4 x 10^8 to 4 x 10^{11} CFU/carcass (Kotula and Pandya, 1995; Lillard, 1989; 1990a) and are reduced to mean $\log_{10}$ 2.5 to 3.7 (Mead and Thomas, 1973; Izat *et. al*., 1988; Lillard 1989; James *et. al*., 1992; Blank and Powell, 1995; Brewer *et. al*., 1995; Cason *et. al*., 1997; Bilgili *et. al*., 2002; Northcutt *et. al*.,

2003; Handley *et. al.*, 2010). Researchers often validate the efficacy of new intervention strategies pre- and post intervention application (Millilo and Ricke, 2010, Millilo *et. al.*, 2011). Industry validates the processing system pre- and post process per regulation (FSIS-USDA, 1996a). An effective means to quantitate the multi-hurdle process is through bio-mapping.

Bio-mapping measures the microbial recovery pre- and post intervention for the whole process. This map will effectively reveal where intervention strategies are successful or failing. In order to determine the effectiveness against potential pathogens, the employment of indicator organism proves useful (Russel, 2000; Whyte *et. al.*, 2004; James *et. al.*, 2006; Handley *et. al.*, 2015; Kim *et. al.*, 2017). For instance, Enterobacteriaceae is a family of bacteria that contains pathogens such *E. coli* O157:H7 and *Salmonella* (Whyte *et. al.*, 2004). Ideally, an indicator organism would be a non-pathogenic microorganism that behaves similarly to the environmental conditions as a target human pathogen.

Currently, there is not an ideal indicator organism that can be utilized to validate new intervention strategies within in a commercial processing abattoir. Recently, microbiome-based 16S sequencing has been applied to samples across the farm to fork continuum and they include both environmental and carcass samples, such as litter, fecal samples, carcass rinsates, and carcass weeps (Oakley *et. al.* 2013; Rothrock *et. al.* 2016; Kim *et. al.* 2017). Next generation sequencing has proven that it can be an effective tool in identifying the microbiomes of complex samples (Ricke *et. al.*, 2015, 2017)

The current study was undertaken to establish next generation sequencing as an applicable tool in conjunction with current quantitative plating techniques used to validate the multi-hurdle interventions employed through the evisceration process. Commercially processed carcass microbiomes were characterized to establish a typical processing microbiome profile and

quantitative data was collected to measure the antimicrobial reduction capacity of rehang to post

chill interventions. Lastly, this study may shed light onto the complex microbiota that includes

both human pathogens and meat spoilage microorganisms on poultry carcasses and may identify

individual bacteria or groups of bacteria as potential indicator organisms.

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Chapter 2

Microbiome of Commercial Broilers Through Evisceration and Immersion Chilling

Introduction

Consumers of both retail and food service establishments today often hear about food recalls due to illness associated with human pathogens either viral or bacterial. Through social media, internet, newspaper, journals, radio and television, consumers have more access to current world events than ever before in history. In 2011, Jill McCluskey and Johan Swinnen reported that news coverage over food and biotechnology products was received through popular press and television outlets of which privately held news agencies focused on shareholders and mass audience expectations (McCluskey and Swinnen, 2011). Therefore, extensive media coverage is provided during these recalls which causes consumers to develop a heightened perception of food being more at risk and amplifies the consequences of the recalled product type. The time interval at which consumers are informed of food recalls can be attributed to the populations' connection to various media platforms, such as social media, internet or television. However, the excessive reporting by various media outlets, can at times, cause a perception of unsafe food; which is simply not true. Food scares are prime examples of how a flood of media coverage can lead to a decline in demand for the product in question, often concomitant with a level of panic that scientists would argue is not appropriate, given the actual risks (McCluskey and Swinnen, 2011). Even though food is far safer today than the first days of food processing, epidemiological evidence indicates that there are still far too many cases of illness to be complacent. According to the *Salmonella* Annual Summary of 2002 from the Center of Disease Control (CDC), 164,044 *Salmonella* infections were reported by the National *Salmonella* Surveillance System from 1998 to 2002; which was approximately 32,000 illnesses annually. In the 2006 *Salmonella* Annual Summary by the CDC, there was approximately 40,666 confirmed

Salmonella cases (CDC, 2006a). In the 2009 *Salmonella* Annual Summary by the CDC, there was approximately 40,828 confirmed *Salmonella* cases. In the more recently published report, the 2013 *Salmonella* Annual Summary by the CDC indicated that there were 45,735 confirmed *Salmonella* cases. It was once estimated that there were 76 million illnesses and 5,000 deaths that occurred each year due to foodborne illnesses, of which *Salmonella* caused an estimated 1.4 million cases (Mead *et. al*., 1999). However, in the more current estimate by Scallan *et.al.* (2011), 37.2 million illnesses occurred in the United States and were caused by 31 pathogens. Of the 37.2 million cases, it was estimated that 9.4 million cases were foodborne related and 1.0 million of those estimated cases was caused by non-typhoidal *Salmonella* spp. As for hospitalization and deaths caused by non-typhoidal *Salmonella* spp., it was estimated that foodborne illness was responsible for 19,336 hospitalizations and 378 deaths.

In regard to the estimated monetary cost of foodborne illness, it has been suggested that the annual health-related cost of all foodborne illness was $51.0 billion and includes medical care, productivity loss, and mortality (Scharff, 2012). Food production companies continue to improve, but there are still no single solutions to achieve complete reduction. In 2009, Buzby and Roberts estimated that annual costs for foodborne illness in the United States were between $6.5 billion to $34.9 billion USD for six bacterial pathogens and one parasite. Previously, Roberts (2007) projected the cost of all foodborne disease to be $1.4 trillion USD.

Another cost to food processors and customers is product spoilage. Buzby *et. al.* (2014) reported food waste in 2010 to be 31% or 133 billion pounds out of 430 billion pounds destined for retail and consumer consumption went un-eaten and was equivalent to $161.6 billion USD. Of the 133 billion pounds wasted, meat, poultry, and fish accounted for 30% or 39.9 billion pounds and $48 billion USD. These types of staggering losses for wasted meat, poultry, and fish

products by food retailers, food service and consumers had been reported previously and was estimated to be as much as 8.5% (8.2 billion pounds) of the 96 billion pounds of lost edible food (Kantor *et. al.*, 1997). The loss of meats accounts for bacterial spoilage, inadequate packaging, temperature abuse, plate waste, and other transportation and mishandling occurrences during distribution due to frequent handling of the commodity (Kantor *et. al*., 1997, Buzby *et. al.*, 2014). The estimated cost of illness and product spoilage impact consumers and food processors. Therefore, it is in the best interest of the food industry to provide their consumers with the highest quality product, which includes providing microbiologically safe food. In order to provide safe and quality products to consumers, meat and poultry firms have aggressively addressed the industry's food safety needs by developing new technologies and sharing these advancements amongst each other, along with the employment of Good Manufacturing Practices (GMPs) and Hazard Analysis Critical Control Points (HACCP) (Kramer *et. al.*, 2005). The GMP and HACCP programs have been implemented and are monitored by the processing firms and government employees to ensure a safe food supply for consumers. In this review, the evisceration process will be explored to better determine factors and interventions that impact the microbial ecology of post chill, eviscerated, whole chicken carcasses as well as how next generation sequencing can be employed to characterize and detect shifts in microbial communities present during evisceration.

Food safety regulations in poultry processing

During the late 1990's, the government initiated HACCP which required plans to be developed by meat processing establishments where the risk analysis included the reduction of pathogens in the processed product. According to the Code of Federal Regulation (CFR), 9 CFR 417.2 (b)(1), "every food processing establishment must develop and implement a written

HACCP plan covering each product produced by that establishment whenever a hazard analysis revealed one or more food safety hazards that are likely to occur" (FSIS-USDA, 1996a). Critical control points are defined as "a point, step, or procedure in a food process at which control can be applied and, as a result, a food safety hazard can be prevented, eliminated, or reduced to acceptable levels" (Ingham, 2007). Additionally, microbiological criteria was included in the original regulation. In the case of poultry meat and the onset of HACCP in 1997, the Food Safety Inspection Service of the United States of America Department of Agriculture (FSIS-USDA) determined that both *Escherichia coli* Biotype I and *Salmonella* would be measured via microbiological methods to measure process control. In 2008, FSIS-USDA released its 2008 progress report indicating a broiler chickens to be 7.3% positive for *Salmonella*, where at the onset of HACCP broilers were 23% for *Salmonella* (FSIS-USDA, 1996b). Therefore, the poultry industry had made significant strides over 12 years in decreasing the incidence of *Salmonella*, but there was still more room for improvement due to the impact associated with illness.

Poultry processors must work with USDA to ensure that both slaughter and raw ground facilities are producing products below the current national *Salmonella* baseline rate (Schlosser *et. al.*, 2000). The previous pathogen reduction performance standards for raw poultry products was 20% positive for *Salmonella* tested by the USDA FSIS, which was a maximum of 12 positive samples out of a set of 51 (FSIS-USDA, 1996b). However, according to the USDA FSIS new performance standards for *Salmonella* and *Campylobacter* (USDA-FSIS, 2010) the performance standard will require processing establishments to have no more than 5 positive samples out of a 51sample set (7.5%) for *Salmonella*. Depending on how often *Salmonella* is recovered from the sample set of 51, processing plants are placed into categories where the

criteria is as follows: Category 1) allows two positives for two consecutive sample sets; Category 2T) allows two positives or fewer in the last set and 3 or more positives in the prior set; Category 2) allows the last set to have 3 to 5 positives and any result for the prior set; Category 3) last set with six or more positives, any result for the prior set (USDA-FSIS, 2010). Processing facilities in Category 1 and 2T will not be posted on the USDA website, but Category 2 and 3 establishments will remain posted for the public.

Also, according to the USDA FSIS performance standards (2010) *Campylobacter* will now be monitored with the performance standard of 1) 8 positive samples using the 1 ml direct plating method per USDA-FSIS Microbiological Laboratory Guidebook (MLG) and 2) 27 qualitative positives out of 51 samples (52%) from either the 1 mL or 30 mL rinsate. The first percentage will be based on a 1 ml sample being directly plated from a 400 mL rinsate, which will yield qualitative and quantitative data. The second percentage will be determined by the 1 ml sample having detectable colony forming units per milliliter (CFU/mL) or a 30 mL sample undergoing an enrichment step, thus allowing a qualitative result for *Campylobacter*.

In addition, each official establishment that slaughters poultry has to demonstrate process control (FSIS-USDA, 1996a). According to 9 CFR 381.94 (a)(3), establishments are required to test for *Escherichia coli* Biotype I using an AOAC approved method at the end of the chilling process with a frequency of 1 sample for every 3,000 carcasses (FSIS-USDA, 1996b). Also, the establishment must report their data in CFU/mL and record the test results on a process control chart or table showing the most recent 13 tests performed. *E. coli* data must be evaluated using statistical process control (SPC) techniques and failure to maintain process control may indicate the inability of the establishment to prevent fecal contamination. In the event of a failure, FSIS will take appropriate action to ensure that the applicable provisions of the law are met.

Harvesting poultry and practices that promote flock colonization by microorganisms

Harvesting broiler meat begins on the farms with live animals being cared for by farmers, veterinarians, and staff dedicated to the well-being of the animals, this stage is referred to as Live Production (Figure 1). The broiler chicken lifecycle begins as an egg being fertilized by the parent flock and then collecting them on site at the breeder farms. The eggs are then delivered to the hatcheries, where they will remain until hatched (Keener *et. al.*, 2004). Once hatched, they are relocated to broiler growout farms until they achieve the desired slaughter weight, typically 6 to 8 weeks (Keener *et. al.*, 2004; Oakley *et. al.*, 2013). Once the broilers are the proper weight for slaughter, they are transported to the slaughter abattoir. A team of individuals, the catching crew, are responsible for collecting the birds on the farm, placing them into transport crates and subsequently onto trucks where they are delivered to the slaughter plant (Clouser *et. al*., 1995; Corry *et. al.,* 2002; Keener *et. al.*, 2004; Oakley *et. al.*, 2013). Upon reception to the slaughter facility, the process of harvesting the chicken meat is as follows (Figure 2): live hang, exsanguination, bleed-out, head removal, de-feathering, feet removal, evisceration, and chilling (Keener *et. al.,* 2004; Handley *et. al.,* 2015). After chilling, the whole carcass will undergo more processing designed to harvest breast, tenders, wings, and thighs for sale as raw parts or taken to further processing for marinade addition, seasoning, or fully cooking (Figure 1) (Handley *et. al.,* 2015). Through this lifecycle, contamination and shifts in the microbial ecology occur through bird to bird or environment to bird contact. These are some of the major contributing factors that influence the microbiota of broiler chickens throughout the farm to fork continuum.

Figure 1. The farm to fork continuum for poultry production begins with live production and end on the consumers' plate.
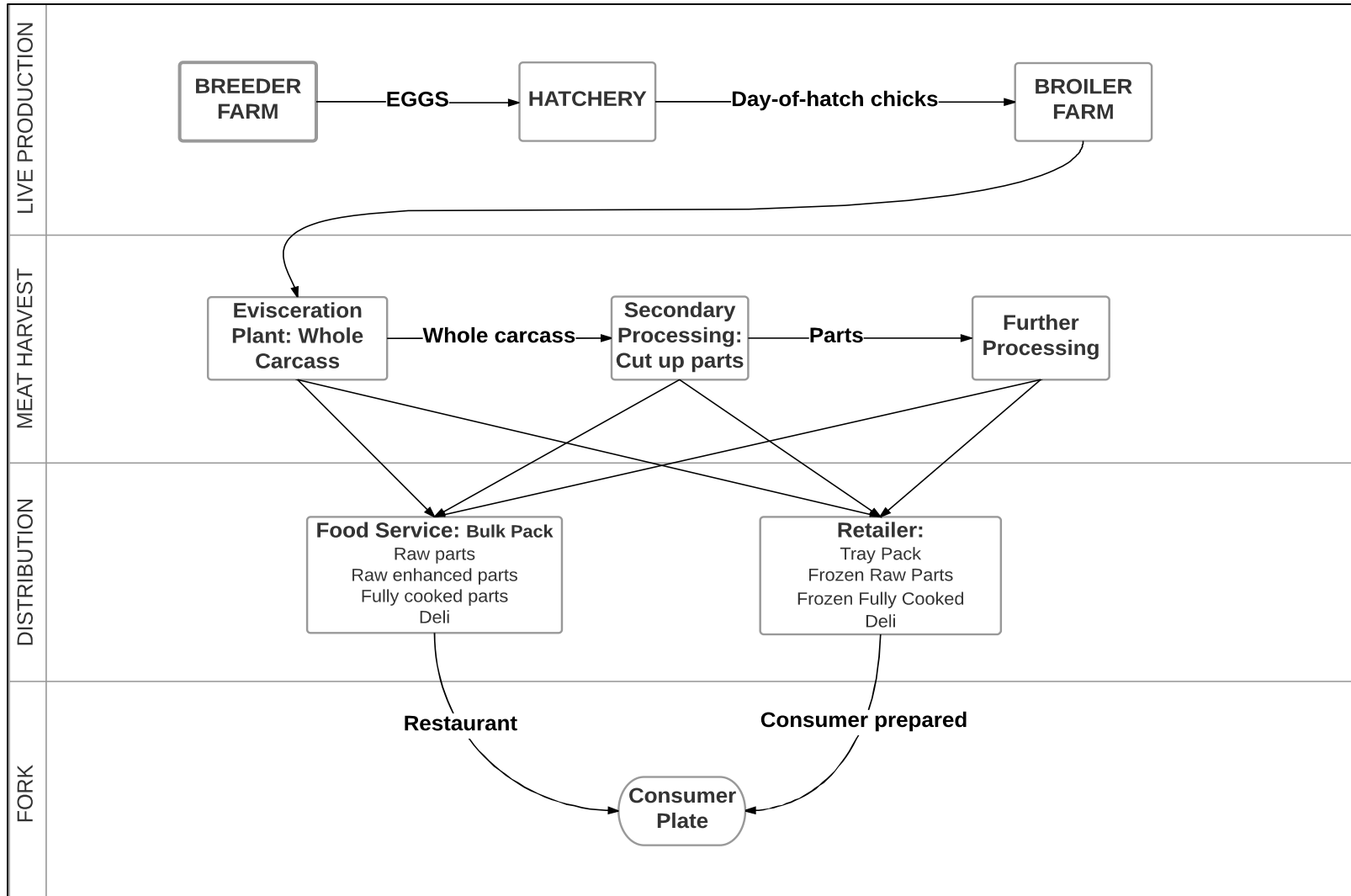
Figure 2. The process flow of poultry evisceration and the stars indicate sampling points.
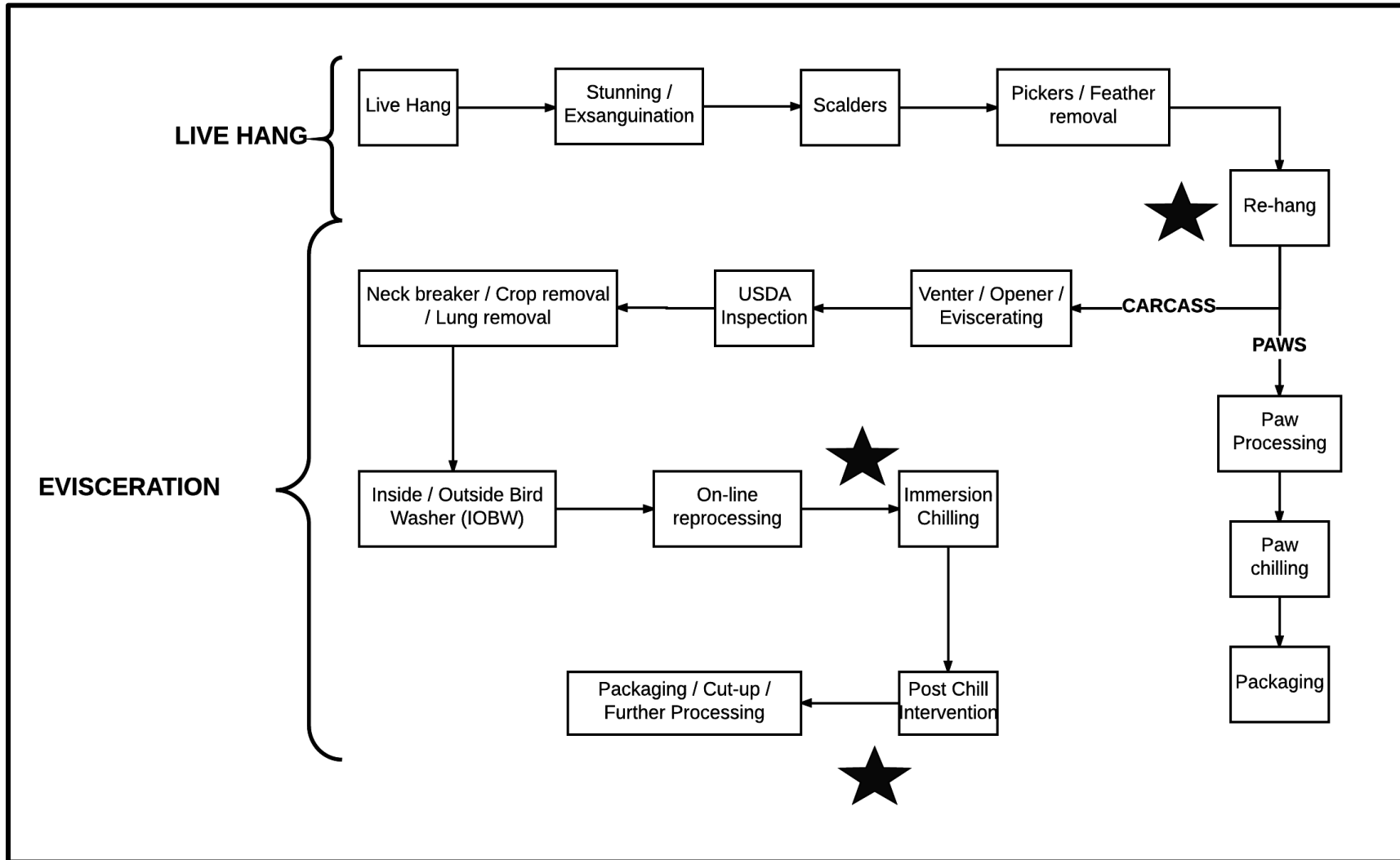
Horizontal Transmission

Poultry are exposed to an array of environmental conditions that contribute to their microbiota and the transmission of bacteria to a chicken or the flock have been described as being either vertical or horizontal transmission (Keener *et. al.*, 2004). Vertical transmission of microorganisms occurs when the infectious agent is passed from the parent to the progeny (Corry *et. al.*, 2002; Cox *et. al.*, 2012). Many studies have successfully demonstrated the concept of vertical transmission through naturally and artificially contaminated breeder hens with *Salmonella* serovars and recovering them in shell eggs (Cox *et. al.*, 2012; Cowden *et. al.*, 1989; Crespo *et. al.*, 2004; Kim *et. al.*, 2007; Lee *et. al.*, 2007; Liljebjelke *et. al.*, 2005; Sanders *et.al.*, 2001; Murase *et. al.*, 2001). As for horizontal transmission, individual broilers or the flock become contaminated through external sources and they include other farm animals, pets, wildlife, insects, rodents, workers, equipment, litter, feed and water (Keener *et. al.*, 2004; Cox *et. al.*, 2012). In regards to the evolving microbiota, vertical transmission can be an originating source for colonization, however, horizontal transmission is a large contributor to shifts in microbiota of one chicken and the flock (Mead, 2004).

Hatchery

During live production, the hatchery offers the first opportunity for bacterial contamination by horizontal transmission in day-of-hatch chicks and has been noted as an important vector for horizontal transfer of microorganisms (Byrd *et. al.*, 1998a; Cox *et. al.*, 1990a, 1991). Eggs from various parent flocks are pooled together at the hatchery; upon hatching, chicks are exposed to potential contaminants from their littermates and the hatchery environment. For example, Cox *et. al.* (1990a) evaluated several commercial hatcheries for *Salmonella* spp. and reported 75% of the environmental samples as *Salmonella* spp. positive;

samples included egg shell fragments, belting material, and chick paper pads. However, various research and surveys of commercial hatcheries have noted that only 5 to 9% of newly hatched chicks leaving the hatchery are positive for *Salmonella* (Byrd *et. al.*, 1998a; Jones *et. al.*, 1991; Cox *et. al.*, 1990b; Bailey *et. al.*, 1987). The low incidence of *Salmonella* spp. for hatched chicks does correlate to percentages found during screenings of table and breeder eggs of which 0.4% to 6.5% reported positive for *Salmonella* Enteritidis (Humphrey *et. al.,* 1989, 1991; Poppe *et. al.*, 1992; Cox *et. al.*, 2012). Therefore, it is possible that the recovered *Salmonella* positive chicks in the hatchery were due to vertical transmission. An additional factor for such a low incidence in the hatchery may be attributed to the required incubation time for a newly hatched chicken to become colonized with enough *Salmonella* cells to be detected. It has been noted that un-colonized chicks become colonized within a week during growout (Byrd *et. al.*, 1998a; Leaney *et. al.*, 1978). Therefore, colonization does require several days to be colonized. Colonization can occur with a limited number of viable *Salmonella* cells as Leaney *et. al.* (1978) demonstrated that as few as 2 CFU of *Salmonella* introduced intercloacally in day-of-hatch chicks could successfully colonize the gastrointestinal tract. Byrd *et. al.* (1998a), reported 100 CFU of *Salmonella* ingested orally as a threshold to cause infection. As for the horizontal transfer of *Campylobacter* at the hatchery level, current research suggests that day-of-hatch chicks are negative for the presence of *Campylobacter* in both samples in the hatchery and the chick themselves (Herman *et. al.*, 2003; Keener *et. al.*, 2004; Cox *et. al.*, 2012; Oakley *et. al.,* 2013) Additionally, day-of-hatch chicks reportedly have a naïve intestinal community where such a nearly sterile environment permits for colonization of a pathogen upon exposure (Byrd *et. al.*, 1998a; Oakley *et. al.*, 2013)

Broiler Farm

During the next stage of live production, the day-old chicks arrive on the growout farm and will remain on the farm until the broilers grow to the desired slaughter weight. During this time on the farm, horizontal transmission is still highly probable. Primary sources of horizontal contamination include the growout house litter, water, external surfaces of equipment and the house interior, insects, rodents, feathers of broilers and boots of farm employees (Keener *et. al.,* 2004; Corrier *et. al.*, 1999; Cox *et. al.,* 2012). For instance, Byrd *et. al*. (1998a) reported as little as 5% of the hatched chicks being exposed to even 100 CFU of *Salmonella* yielded a more than 50% infection of broilers in the growout house. Once a broiler has been infected with *Salmonella*, it can be shed through feces where other chickens encounter *Salmonella* in the broiler house litter. Instances of litter contaminated with *Salmonella* have been well documented (Byrd *et. al.*, 1998a; Corrier *et. al.,*1999). *Campylobacter* has also been identified in litter (Herman *et. al.,* 2003; Keener *et. al.,* 2004; Cox *et. al.,* 2012). In a *Campylobacter* assessment from hatchery to slaughter, *Campylobacter* was not recovered in either the one day old chicks or the growout farm, but the percent positive rate increased over seven weeks of sampling to 90% *Campylobacter* positive (Herman *et. al.,* 2003). Others have reported similar horizontal transfer of *Campylobacter* infection to the flock as the duration of time increased on the growout farm (Keener *et. al.,* 2004; Cox *et. al.,* 2012). Water was also a vector contamination, as Herman *et. al.* (2003) found contamination occurred in 5 of the 6 flocks. Water has served as a carrier that has enabled *Campylobacter* to spread (Keener *et. al.,* 2004; Cox *et. al.,* 2012).

Feed withdrawal

This stage of the growout process is typically the last hours on the farm prior to being transported to the slaughter facility. Feed withdrawal requires farmers to remove all feed from

the growout house so that there is minimal to no ingesta present during the slaughter process; however, water does remain available to prevent dehydration of the chickens. Feed withdrawal has been established to minimize the amount of fecal material coming into the production facility. According to 9 CFR 381.65, "Poultry carcasses contaminated with visible fecal material shall be prevented from entering the chilling tank" (USDA-FSIS, 1972a). Additionally, carcasses or tissues that are contaminated with digesta or feces from the crop, cloaca, or due to a torn or cut gastrointestinal tract (GIT) will result in condemnation or trimming of the carcass (Doyle and Erickson, 2006; USDA-FSIS, 1972c). Therefore, it is necessary to perform feed withdrawal to prevent the un-necessary trimming and or condemnation of carcasses contaminated with ingesta or feces.

Feed withdrawal typically occurs between 8 and 12 hours prior to evisceration. During this period of feed deprivation, physiological changes occur in the chicken GIT. These physiological changes increase the potential for horizontal transmission of bacterial communities. The practice of feed withdrawal has shown to impact the crop significantly as it relates as a vector for carcass contamination during processing (Hargis *et. al*., 1995; Humphrey *et. al.,* 1993; Ramirez *et. al.,*1997; Corrier *et. al.,* 1999). The crop is a nonsecretory organ in which food is stored prior to passage into the gizzard (Durant *et. al.*, 1999; Fuller and Brooker, 1974). It has a pH of approximately 5.0 due to the colonization of Lactobacilli in the stratified squamous epithelium of the crop; however, evidence suggests that the feed withdrawal process diminishes the number of colonized Lactobacilli (Durant *et. al.*, 1999; Hinton *et.al*., 2000). In a challenge, where crops of laying hens were assessed during a feed withdrawal induced molt, the pH of the crop increased from near 5.0 to 6.2 (Durant *et. al.*, 1999). Several research studies have identified the role in which pH can influence shifts in the microbiota of the crop (Durant *et. al.*,

1999; Hinton *et. al.*, 2000; Keener *et. al.*, 2004; Doyle and Erickson, 2006). Feed withdrawal studies have demonstrated the colonization and increase of *Salmonella* and *Campylobacter* incidence in the crop (Humphrey *et. al.,* 1993; Ramirez *et. al.,*1997; Byrd *et. al.*, 1998b; Corrier *et. al.,* 1999; Hinton *et. al.*, 2000) Corrier *et. al.* (1999) reported a 5.3-fold increase in the prevalence of *Salmonella* positive crop from before and after feed withdrawal. Ramirez *et. al.* (1997) noted that *Salmonella* was greater after 8 hours of feed withdrawal than before undergoing feed withdrawal. Byrd *et. al.* (1998b) recorded a 45% increase in *Salmonella* incidence while evaluating pre- and post feed withdrawal effects, 90 out of 360 birds or 25% and 254 out of 359 birds or 70% respectively. These studies are analogous of other feed withdrawal trials in regards to shifts in microbiota. As for the root cause, Corrier *et. al.* (1999) observed and recorded an increase in pecking activity of the flock pre- to post feed removal which also correlated to a higher *Salmonella* crop incidence for those broiler houses with positive *Salmonella* drag swabs. As poultry commonly forage for food, it does seem plausible for colonization to occur in the crop seeing as the organisms may have been recently consumed during the feed withdrawal process.

The ceca has also been studied extensively during feed withdrawal challenges and the assessments indicate it as a potential vector of contamination during processing, but at a relatively infrequent occurrence (Hargis *et. al.,* 1995). For instance, Corrier *et. al.,* (1999*)* reported a lack of significant increase in ceca *Salmonella* positive broilers before (14/240 or 5.8%) and after (19/240 or 7.9%) feed withdrawal. Hargis *et. al.* (1995) examined both ceca and crops during the evisceration process and found that 6 of 2,100 (0.3%) ceca were ruptured whereas 48 of 187 (25.7%) crops collected were ruptured. They also sampled 550 crops and 500 ceca of 3 commercial flocks prior to evisceration for *Salmonella*; crops for plant 1, 2, and 3 were

62%, 16% and 86.7% positive, while the ceca were 12.5%, 16.5%, and 14.6%, respectively (Hargis *et. al.,* 1995). Ramirez *et. al.* (1997) noted that 8 hours of feed withdrawal exhibited a minimal impact in *Salmonella* colonization of the ceca for commercial broilers. Humphrey *et. al.* (1993) reported that feed withdrawal slowed down colonization of the ceca in Leghorn laying hens orally administered 10^6 *Salmonella* Enteritidis; however, survival increased in the crop. While the ceca certainly harbors *Salmonella,* colonization does not occur as rapidly as the crop and the physical structure of the organ does not seem to be compromised as easily during the evisceration process.

The intestines also pose another risk factor in the contamination of other broilers and evisceration equipment due to feed withdrawal. The physical integrity of the GIT and its contents, ingesta and feces, become altered during the withdrawal time (Bolder, 2007). Russell (2000) stated the importance of proper feed withdrawal practices, where a shortened (less than 8 hours) withdrawal time would not allow the intestines to become emptied and full intestines are subject to breaking, allowing ingesta to leak on the carcasses; conversely, long (greater than 12 hours) withdrawal causes the intestines to slough the mucosal lining and to develop gas, providing a weakened state that may tear more easily or when cut allow any remaining feces to explode. Northcutt *et. al.* (1997) evaluated the physical attributes of poultry viscera after feed withdrawal times of 0, 3, 9, 12, 14, 16, and 18 hours; the results demonstrated substantial intestinal sloughing and bacterial fermentation for sampling times greater than 12 hours. Bolder (2007) discussed similar points in that watery intestines can lead to a leakage in the contents and a decrease in GIT integrity where the probability of intestinal damage and spillage of digesta occur during evisceration. Fluid contents in the intestines have been shown to increase (Warriss *et. al.*, 2004). Proper feed withdrawal will lead to an overall reduction in the potential

contamination of a carcass, adjacent carcasses, and machinery by limiting the amount of fecal material into the processing abattoir. However, the practice is also contributes to the horizontal transfer of bacteria leading to shifts in the microbiota.

Transportation

This processing step is the last phase of the live production continuum and is another potential vector for flock contamination. During transportation, the catching crew must handle the live animals to place them into crates and handling live animals has been identified as a stressor resulting in excessive shedding of fecal matter (Bolder, 2007). Additionally, increased stress and dehydration during transport has been associated with increased shedding of fecal material (Corry *et. al*., 2002; Bolder, 2007). Lastly, the broilers are still undergoing feed withdrawal; therefore, it is plausible that transportation exacerbates the increased shedding already occurring during feed withdrawal.

With the presence of fecal material on, in, or around the transport crates, transport containers themselves can pose a potential threat toward contaminating live poultry, as Lillard (1989) reported *Salmonella* being isolated from the breasts of caged broilers arriving for slaughter. Clouser *et. al*. (1995) identified transport crates as a vector of cross-contamination for the flock being transported and Rasschaert *et. al.* (2007) reported 11% of the transport containers sampled being contaminated with *Salmonella.* In an investigation of crate cleaning efficacy, Corry *et. al*. (2002) noted contamination of cleaned transport crates with *Salmonella* serovars not isolated from either the farm or the flock that was surveyed during transportation. Contamination of the crates was attributed to 3 findings related to poor cleaning procedures: 1) one plant used waste water to clean the crates, 2) disinfectants were applied in lower than recommended concentrations, and 3) fecal material was visibly observed post cleaning and disinfection.

Therefore, *Salmonella* isolated from the crates were likely from flocks previously transported for slaughter.

Modes of cross contamination within the processing abattoir.

Upon arrival of live poultry to the slaughter facility, more sources of potential microbial contamination can occur. Sources of cross contamination may include bird to bird, flock to flock, processing equipment, processing water and even personnel. As the birds enter the slaughter abattoir, they are usually filthy with dirt, litter, and fecal matter that they may have encountered during rearing and transportation (Mead, 1974; Owens *et. al.*, 2010). Researchers have reported that the skin and feathers are contaminated with feces upon arrival to the slaughter house (Kotula and Pandya, 1995; Jorgensen *et. al.*, 2002; Franchin *et. al.*, 2005) and the fecal matter has been identified as a source of *Salmonella* contamination on caged bird breasts (Lillard, 1989). The farm environment and the transportation process impact the microbiota present on broiler destined for evisceration and feathers contribute to horizontal transmission of microorganisms into the processing plant.

During evisceration, live birds will undergo exsanguination and then proceed to a series of mechanical processes (Figure 2) to remove the blood, feathers, and internal organs, respectively. Water is commonly employed to wash carcasses during the evisceration process, as it is used for scalding, chilling, evisceration equipment, sanitation and is used to dilute concentrated antimicrobials prior to their application on carcasses (Owens *et. al.*, 2010; Handley *et. al.*, 2015; Blevins *et. al.*, 2017). As these are the major elements in a processing facility, cross-contamination has been commonly associated with scalding, plucking, evisceration, and chilling of carcasses (Carrasco *et. al.*, 2012). Many researchers have previously investigated evisceration equipment, processing water, and personnel to better understand the movement of

bacteria through the slaughter facility and the impact on the microbial quality of carcasses (Lillard, 1990b; James *et. al.*, 1992; Sarlin *et. al.*, 1998; Cason and Hinton, 2006; Rasschaert *et al*. 2007; Handley *et. al.*, 2010; Oakley *et. al.*, 2013; Kim *et. al.*, 2017).

Scalder

After the birds are stunned, exsanguinated and bleed out, they undergo scalding. Scalding prepares the feather follicles to allow more efficient feather removal by mechanical pickers and it can remove the cuticle depending on the temperature of the scald water temperature (Blevins *et. al.*, 2017; Bowker *et. al.*, 2014). Hard scalding is the practice of placing the birds through water temperatures of 145°F (62.7°C) to 160°F (71.1°C) and soft scalding occurs at temperatures between 120°F (48.8°C) to 135°F (57.2°C) (Buhr *et. al.*, 2005; Bowker *et. al.*, 2014; Blevins). Most in the U.S are a combination of both (Buhr *et. al.*, 2005; Cason *et. al.*, 1999; Cason and Hinton, 2006; Blevins *et. al.*, 2017). The scalder has been designed as a series of tanks in which water is counterflowed, such that the clean water inlets are at the end of a tank so birds go from dirty water to clean water (Cason and Hinton, 2006; Blevins *et. al*, 2017). These tanks are considered a risk for cross-contamination by pathogens, such as *Salmonella* and *Campylobacter,* as the tanks are not typically drained during production and birds from subsequent flocks continue to be processed during the day (Genigeorgis *et. al*, 1986; Oosterom *et. al.*, 1983; Whyte *et. al.*, 2004). However, Whyte *et. al*. (2004) observed that levels of contamination in the scalder do not continuously increase during the subsequent passage of birds through the tank. Also, assessment of three-stage scalders reported up to 3.0 log reduction from the first to last stage scalder, thus less contaminated carcasses from the multi-tank scalder design as opposed to a single-stage tank (Buhr *et. al.*, 2005; Cason *et. al.*, 1999; Cason and Hinton, 2006; Blevins *et. al.*, 2017). Evidence suggests that the bacterial level is reduced during scalding,

however, it is still a means of microbial translocation from one bird to another and from one flock to another.

Immersion Chiller

The immersion chiller is another step that provides an opportunity for carcasses to become cross-contaminated. The purpose of using the chiller is to immediately reduce the temperature of whole carcasses, parts, and giblets after evisceration so there is no increase in pathogens (USDA-FSIS 1972b). Chilling is carried out in large tanks of cold water with an added antimicrobial and must be monitored closely by the slaughter establishment so as to maintain regulatory compliance with USDA (Blevins *et. al.*, 2017). The chiller allows whole carcasses to encounter an antimicrobial with a dwell time ranging from 60 to 120 minutes, providing an optimal application to reduce the microbial load (Stopforth *et. al*, 2007). Immersion chilling has been noted as being a successful mode of carcass decontamination (Mead, 2004), but has also been criticized for cross-contamination potential (Lillard, 1990b; James *et. al.*, 1992; Sarlin *et. al.*, 1998; Bilgili *et.al.*, 2002; Carraco *et. al*, 2012; Blevins *et. al*, 2017) Water immersion has been recorded as being able to reduce the microbial load of coliform and *E. coli* by as much as 2.5 $\log_{10}$ CFU/mL (James *et. al.*, 2006). Conversely, Brewer *et. al*. (1995) observed a microbial reduction as little as 0.5 $\log_{10}$ CFU/mL while examining the effects of various line speeds on chiller efficacy; no impact from line speed was established. Lillard (1990b) evaluated the chilling process and recorded a washing effect resulting in a mean 0.91 $\log_{10}$ CFU/carcass reduction in Plant A and mean 0.73 $\log_{10}$ CFU/carcass reduction in Plant B for Aerobic Plate Count (APC); as for *Salmonella* incidence it went up from pre-chill to post chill, 15% and 28%, respectively. While the chiller has proven a capable and effective means in

reducing the microbial load, it equally contributes to a re-distribution effect of carcass microbiota to other broilers and flocks.

Equipment

Processing equipment for a large poultry processor could contact between 150,000 to 250,000 head between full sanitation cycles where the equipment is fully broken down for daily cleaning (Rothrock *et. al.*, 2016). Therefore, processing equipment becomes an important mode of transmission between birds and flocks. In a survey of the evisceration environment and equipment, Rasschaert *et al*. (2007) found the plucking and scalding areas yielded a higher incidence of contamination than the evisceration room prior to slaughter activities. These findings seem appropriate being that the scalding and picking room are the location in which feathers are removed and is segregated from evisceration department. Feather removal is accomplished by using scalders and pickers, finger-like rubber attachments that massage the carcass to remove the feathers from the feather follicle (Blevins *et. al.*, 2017). Both steps are required for feather removal, but certainly are points of microbial contamination throughout the production day (Berrang and Dickens, 2000). Additionally, the environmental conditions in the picking area are conducive to supporting microbial persistence due to the moist and warmer room conditions.

In a study comparing the *Salmonella* positive rates between birds contaminated with fecal material and those without, reported a 20.0% incidence for non-fecal birds and a 20.8% incidence for fecal contaminated birds (Jimenez, 2002). The two sample types exhibited nearly the same percent positive results, which may indicate cross-contamination by processing equipment during evisceration since the samples were pulled from a single flock. Also, research has shown that equipment can be positive for *Salmonella* after sanitation and possibly after

multiple rounds of sanitation. Such was the case with Rasschaert *et. al.* (2007) when a

Salmonella negative flock tested positive for *Salmonella* spp. isolated from a known positive

Salmonella positive flock, processed days earlier. Olsen *et. al.* (2003) reported some *Salmonella*

strains can survive up to 5 days in the commercial slaughter environment despite the daily

cleaning and disinfection procedures. It is probable that the contaminated equipment would

immediately effect the first carcasses entering evisceration, demonstrating both bird to bird and

flock to flock contamination.

Biofilms

Contamination of equipment with microorganisms that should have been eliminated

during a cycle of sanitation may be due to their refuge in biofilms. Biofilm formations offer

bacterial cells a protective barrier to grow and survive in harsh environmental conditions, such as

sanitation (Reuter *et. al.*, 2010; Carrasco *et. al.*, 2012; Steenackers *et. al.*, 2012). It has been

reported that *Salmonella* has the capability of attaching itself to inert surfaces in the food

processing environment and producing biofilms (Hood and Zottola, 1997; Joseph *et. al.*, 2001;

Stepanovic *et. al.*, 2004; Carrasco *et. al.*, 2012). Biofilms are characterized as bacterial cells or

communities encapsulated in an exopolysaccharide matrix which enables cells to adhere to one

another and to surfaces (Costerton *et. al.*, 1987; Chmielewski and Frank, 2003; Hood and

Zottola, 1995). This exopolysaccharide matrix is also known as an extracellular polymeric

substances (EPS) which contain polysaccharides, proteins, phospholipids, teichoic and nucleic

acids, and other polymeric substances hydrated with 85 to 95% water (Costerton *et. al.*, 1987;

Sutherland, 1983; Chmielewski and Frank, 2003). Cells aggregate to form micro-colonies

enclosed within a hydrated, predominately anionic, matrix with pores or channels throughout the

structure to allow transportation of oxygen, nutrients and waste (Costerton *et. al.*, 1987; Sofos, 2010).

Current research has attributed biofilm formation to the expression of genes producing curli fibers, exopolysaccharide, and cellulose (de Rezende *et. al*., 2005; Solomon *et. al.*, 2005; Kim and Wei, 2009; Steenackers *et. al.,* 2012). Depending on the surface at which these biofilms attach, the structural composition may vary. For instance, biofilm development on glass surfaces and gallstones differed in flagellum expression, lipopolysaccharide (LPS) and exopolymeric substance composition (Kim and Wei, 2009). Additionally, gene knock out studies have demonstrated that mutants are more susceptible to desiccation; these mutants lacked genes associated with the regulation of cellulose, O-Ag-capsule, curli fimbriae and other *Csg*D-regulated components (White *et. al*., 2006; Gibson *et. al.*, 2006; Steenackers *et. al.,* 2012) Biofilm forming bacteria have been associated with both pathogens and spoilage microorganisms, such as *Listeria, Salmonella, Campylobacter, E. coli*, *Pseudomonas* and lactic acid producing bacteria, and they may be dominated by one specie or a mixed culture (Chmielewski and Frank, 2003; Hood and Zottola, 1995).

Regardless of bacterial species present, the cell matrices form a network which facilitates formation and maintenance of the biofilm structure, and increases the resistance of biofilms to sanitizers (Costerton *et. al.*, 1987; Chmielewski and Frank, 2003; Sofos, 2010). It has been noted that *Salmonella* biofilms can be formed on plastic, cement, and stainless steel and are more resistant to sanitizers than their planktonic counterpart (Joseph *et. al.*, 2001). Sofos (2010) noted that biofilm associated bacteria could be up to 500 times more resistant to sanitizers, such as lactic acid, quaternary ammonium, sodium hypochlorite or hydrogen peroxide-based, than their free flowing planktonic counterparts; in some cases, a marginal increase of 10 to 100-fold in

concentration level or exposure time may be effective against biofilm associated bacteria as compared to planktonic cells. Such was the case with Joseph *et. al.* (2001), where two sanitizers, 100 ppm chlorine solution and 50 ppm iodine, removed the biofilm after 15 minutes' contact time as opposed to planktonic cells being susceptible with either 10 ppm of chlorine or 10 ppm of iodine and contact time of 10 and 5 minutes respectively (Steenackers *et. al.*, 2012). Conversely, Moretro *et. al.* (2009) noted 400 ppm of hypochlorite on stainless steel for 5 minutes was not sufficient for the removal of *Salmonella* biofilms (Steenackers *et. al.*, 2012). Increased resistance may be due to a smaller surface area exposed to sanitizers and potentially the expression of genes associated with sanitizer resistance (Sofos, 2010). Clearly, biofilms and the bacteria associated with them provide an opportunity for horizontal transmission on equipment and considered in the contribution to microbiota of post chill carcasses.

Personnel

Another source of contamination can be from employees and their practices during a typical working shift (Todd *et. al.,* 2010; Carrasco *et. al.*, 2012). Personnel can transfer bacteria throughout a production facility. If the employee by-passes certain interventions dedicated to preventing cross-contamination, then they would contribute to the contamination of an otherwise clean environment. Contamination of food products have been traced back to hands, gloves, dirty clothes, and coughing. The CDC (2006b) has ranked hands as a high-risk factor associated with outbreak investigations. As early as 1938 Price reported bacterial counts on the area from the hands to 2 inches above the elbow to be between 2×10^6 and 1×10^7 CFU total aerobic plate count with 90% of these organisms residing on the hand (Todd *et. al.*, 2010). Taylor *et. al.* (2000), recovered bacterial counts of 10^2 to 10^6 CFU/hand swab samples (Todd *et. al.*, 2010).

Recommendations in the FDA Food Code (2013) guidance document emphasizes the

benefits of using gloves. While gloves will reduce the transmission of bacteria from employee hands to the food, gloves will only be effective so long as they are maintained in a sanitary manner (Todd *et. al.*, 2010). More recently, Pellegrino (2015) found that posters emphasizing proper hand washing may be ineffective in hand washing habits. Rather their observations noted that smell increased the probability of hand washing compared to the control, auditory, and visual ques (Pellegrino *et. al.*, 2015). Therefore, utilizing the olfactory sense to improve the hygiene practices of personnel in conjunction with training could reduce the horizontal transmission of bacteria.

Factors contributing to the persistence of microorganisms in processing abattoirs

During the rearing, transportation, and evisceration process, bacteria are subjected to a variety of conditions that may induce the upregulation and deregulation of genes that prepare them for surviving harsh conditions. Feed withdrawal and transportation, discussed previously, stress the birds and disrupts normal intestinal functions; subjecting the animal to opportunistic pathogens (Keener *et. al.*, 2004). Since the digestive tract is experiencing increased shedding of microorganisms, bacteria are exposed to environmental conditions of digestive tract which include a lower pH, the presence of volatile fatty acids, and lower oxidation-reduction potential (Ricke, 2003b). These new environmental stresses may aid in the adaptation of resistance to more lethal concentrations of antimicrobials present during evisceration. Microbial resistance to an antimicrobial can occur when a sub-lethal dosage is encountered, as it is a cellular response to increase protective mechanisms for that environment which may lead to protection from a similar antimicrobial (Kwon and Ricke, 1998; Kwon *et al.*, 2000; Leyer and Johnson, 1993; Rishi *et. al.*, 2005; Calhoun and Kwon, 2010). Another reason may simply be due to the numbers of organisms present. If there are 1000 cells and the multiple hurdle in the process can only

remove 95% of all organisms, then there are still 50 viable cells remaining. Lastly, survival may be enhanced by a cells ability to fully attachment itself to the skin or equipment surface (Lillard, 1989).

Genetic coding either on the extrachromosomal genetic element or on a plasmid is a way cells can survive acid treatments (Ricke, 2003a). This type of resistance is possibly passed to other organisms of the same species or populations of genetically different organisms and has been well documented in antibiotic and inorganic chemical-based disinfectants (Ricke, 2003a). One mechanism to pass on genetic information is for the cell to take up a plasmid followed by subsequent replication during microbial multiplication (Davies, 1994; Koutsolioutsou *et. al.*, 2001). An example of such a system that would enable a microorganism to survive acidic conditions is the acid tolerance response (ATR) system (Kwon and Ricke, 1998). Such a system reportedly becomes more robust with longer exposure to short chain SCFA, anaerobiosis, and acid pH (Kwon and Ricke, 1998), which all three conditions exist in the gut. The ATR system is seen in both Gram positive and Gram negative organisms and is an environmental stress response system that enables cells to survive pH values as low as pH 3 (Park *et. al.*, 1996; Brul and Coote, 1999). Other research has demonstrated that *Salmonella* Typhimurium can survive up to 4 hours under acidic conditions at approximately pH 3.0 after the initiation of the ATR system (Rishi *et. al.*, 2005).

Additionally, it may explain how *Salmonella* and *Campylobacter* could survive in the previously described feed withdrawal studies. The induction of ATR allows for the transcription of genes responsible for synthesizing a series of acid shock proteins that enable cells to maintain homeostasis under extreme acidic conditions that would normally be lethal (Foster, 1999; Ricke, 2003a). Treatment of organic acids at mild to neutral pH have shown to be an environmental

stimuli for ATR and provide further protection against more extreme acidic conditions. *Listeria monocytogenes* possesses the ability to utilize an acid tolerance response at pH 3 after prior exposure to pH 5.0 (Davies *et. al.*, 1996 Brul and Coote, 1999). High concentrations of short chain fatty acids at neutral pH have been found to provide an ATR in both *E. coli* and *S. typhimurium* (Kwon and Ricke, 1998; Arnold *et. al.*, 2001). Kwon and Ricke (1998) also noted that inorganic acid resistance of *S.* Typhimurium increased after exposure to high concentrations of SCFA and was further enhanced by acidic pH, anaerobiosis, and prolonged exposure to the SCFA (Ricke 2003a). Conversely, *E. coli* O157:H7 demonstrated resistance against benzoic acid once treated with a strong acid at pH 2.0 (Lin *et. al.*, 1996; Brul and Coote, 1999). It is evident that increased resistance to acid conditions are likely to be induced in the food processing systems.

Another mechanism of resistance may be a metabolically transient or expressed system that excludes the antimicrobial compound from interacting with the cell by breaking it down extracellularly, secreting it from within the cell, or detoxifying itself by utilizing it in a metabolic pathway. An example of detoxification would be the glutamate decarboxylase (GAD) system where the cell reduces the amount of protons present within the cytoplasm. The GAD system operates by catalyzing the irreversible reaction of an extracellular glutamate to one extracellular gamma-aminobutyrate and one CO_2 by consuming an internal proton (Alonso-Hernando *et. al.*, 2009). Another mode of action employed by cells against an extracellular compound was noted by Hugo and Foster (1964) where *Pseudomonas aeruginosa* employed a specific extracellular esterase enzyme to degrade methyl *para* (4)-hydroxybenzoate (methylparaben), a food preservative, to methyl alcohol and 4-hydroxybenzoic acid (Brul and Coote, 1999). Valkova *et. al.* (2002) demonstrated that resistance could be transmitted to other organisms not known to

have resistance to parabens by inserting the gene *prbA*. Under aerobic conditions, the enzyme ρ-hydroxybenzoate hydroxylase catalyzes the hydroxylation of ρ-hydroxybenzoate to form protocatechuic acid, also known as 3,4-dihydroxybenzoic acid (Cole *et. al.*, 2005) where it can then be further metabolized utilizing the β-ketoadipate pathway (Valkova *et. al.*, 2001). Under anaerobic conditions, ρ-hydroxybenzoic acid is converted into a phenol by a decarboxylase (Valkova *et. al.*, 2001). However, Valkova *et. al.* (2001) demonstrated the ability of *Enterobacter cloacae* to break down methylparaben to a phenol via the anaerobic pathway under aerobic conditions and was resistant up to 800 mg/liter of methylparaben. Regardless of the method to resist antimicrobial and toxic compounds, organisms have been identified that can utilize extracellular enzymes and other metabolic pathways to break down inhibitors and or prevent the acidification of the inner cell.

Cell attachment

Pathogens and spoilage organisms are recovered in small numbers from the poultry product after the chilling process. A plausible explanation for this phenomenon has been investigated by Lillard (1989) which concluded that *Salmonella* cells present on the skin were initially entrapped in a water film on the skin and then migrated to the skin; where they were entrapped in ridges and crevices that became more pronounced in the skin after immersion in water. Prior to this conclusion, chicken skin was submersed in a cellular suspension of *Salmonella* Typhimurium for 15 seconds, 30 minutes, and 60 minutes where 90% of cells were recovered in the water film or less than 10% absorbed by the skin; the 30 minute and 60 minute immersion times recovered 40% and 60% of bacterial cells from the skin, respectively (Lillard, 1989). Additionally, confirmation of cellular attachment was concluded by performing scanning electron microscopy (Lillard, 1989) and was in agreement with other published research

(Notermans and Kampelmacher, 1974; McMeekin and Thomas, 1978). The ability to bind onto chicken skin was also demonstrated with *E. coli*, *Lactobacillus brevis, Klebsiella* sp., and three *Pseudomonas* ssp. and adherence was optimal at 20 ºC (Notermans and Kampelmacher, 1974). It remains clear that bacterial cells do become more closely associated with the skin as time increases and the greater the time allowed for microbial attachment the more firmly attached they become. Evidence of this was demonstrated by Lillard (1989) where aerobic bacteria and *Enterobacteriaceae* were recovered after 40 consecutive whole bird carcass rinses.

Currently, there are two theories for bacterial attachment to meat where one is either thought to be a two-stage process (Figure 3A, 3B) (Marshall *et. al.*, 1971) or a three-stage process (Figure 3C) (Busscher and Weerkamp, 1987). The two-stage process consists of reversible and irreversible attachment (Marshall *et. al.*, 1971; Selgas *et. al.*, 1993). Reversible attachment occurs when bacteria become entrapped in a water film on the contact surface (Selgas *et. al.*, 1993). In addition, Hood and Zottola (1995) noted that the bacteria can be removed by simply washing. Irreversible attachment is described as a more permanent physical attachment of the bacterial cell to the surface (Selgas *et. al.*, 1993) and involves the production of an extracellular polysaccharide (Hood and Zottola, 1995). The other cellular attachment theory was proposed by Busscher and Weerkemp (1987) and consists of a three-stage process. The first stage only involves long range forces such as electrostatic charges and van der Waals forces that occur at distances greater than 50 nm. The second stage begins as the microorganism approaches 20 nm and is reversible at first, but as time progresses it becomes irreversible. The third stage occurs at distances less than 15 nm and involves adhesive polymers produced by bacterial cells (Hood and Zottola, 1995). Regardless of the proposed model for bacterial attachment to surfaces, physiochemical forces such as electrostatic charge and van der Waals forces are only part of the

equation. Bacterial cells must be able to sustain the permanent bond between the inert surface in which the bacteria has affixed themselves.

Figure 3A. Two-stage process - Reversible attachment of bacteria entrapped in water. Adapted from Marshall *et al.* (1971)
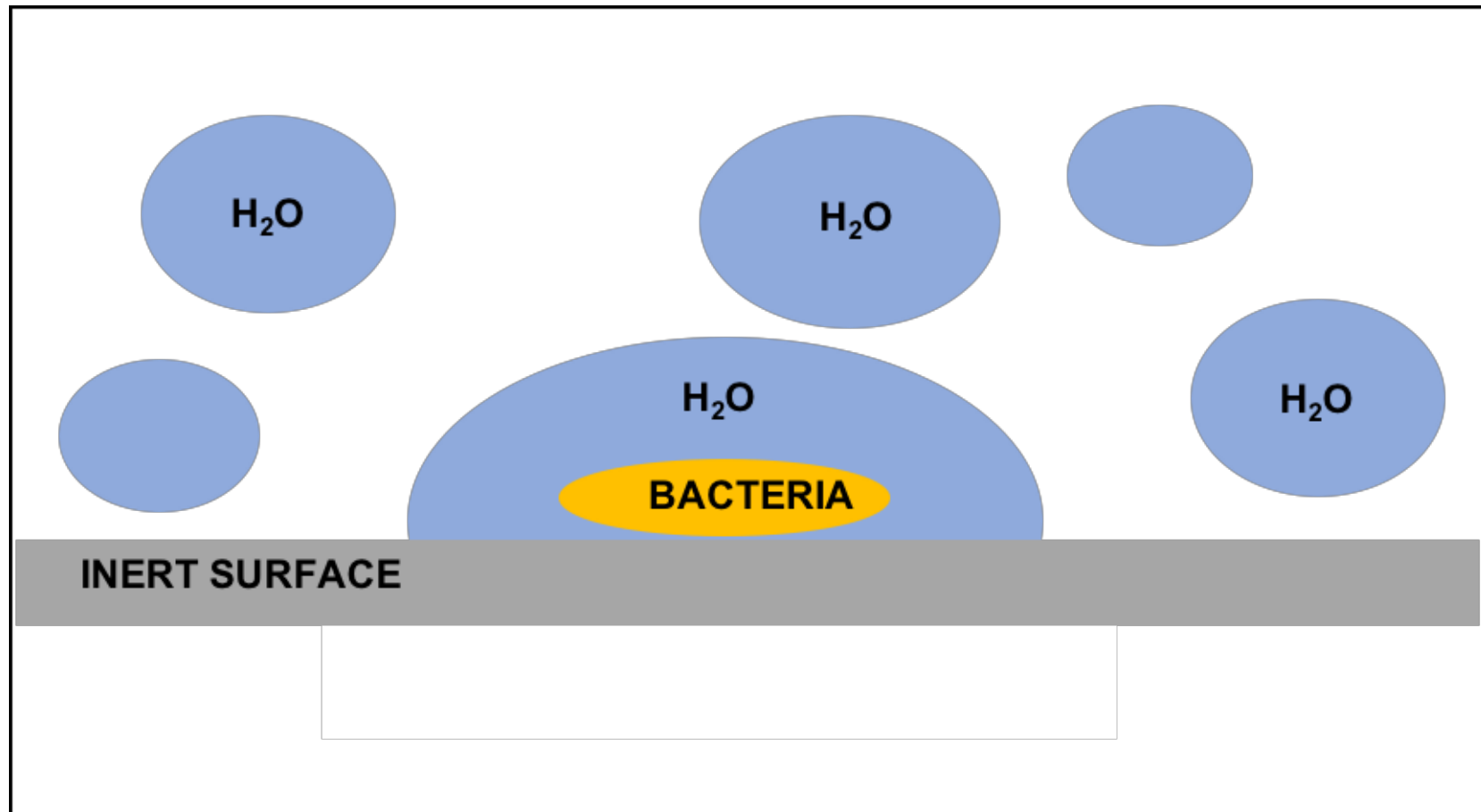
Figure 3B. Two-stage process - Irreversible attachment of bacteria due to extracellular polysaccharide adapted from Marshall *et al.* (1971).
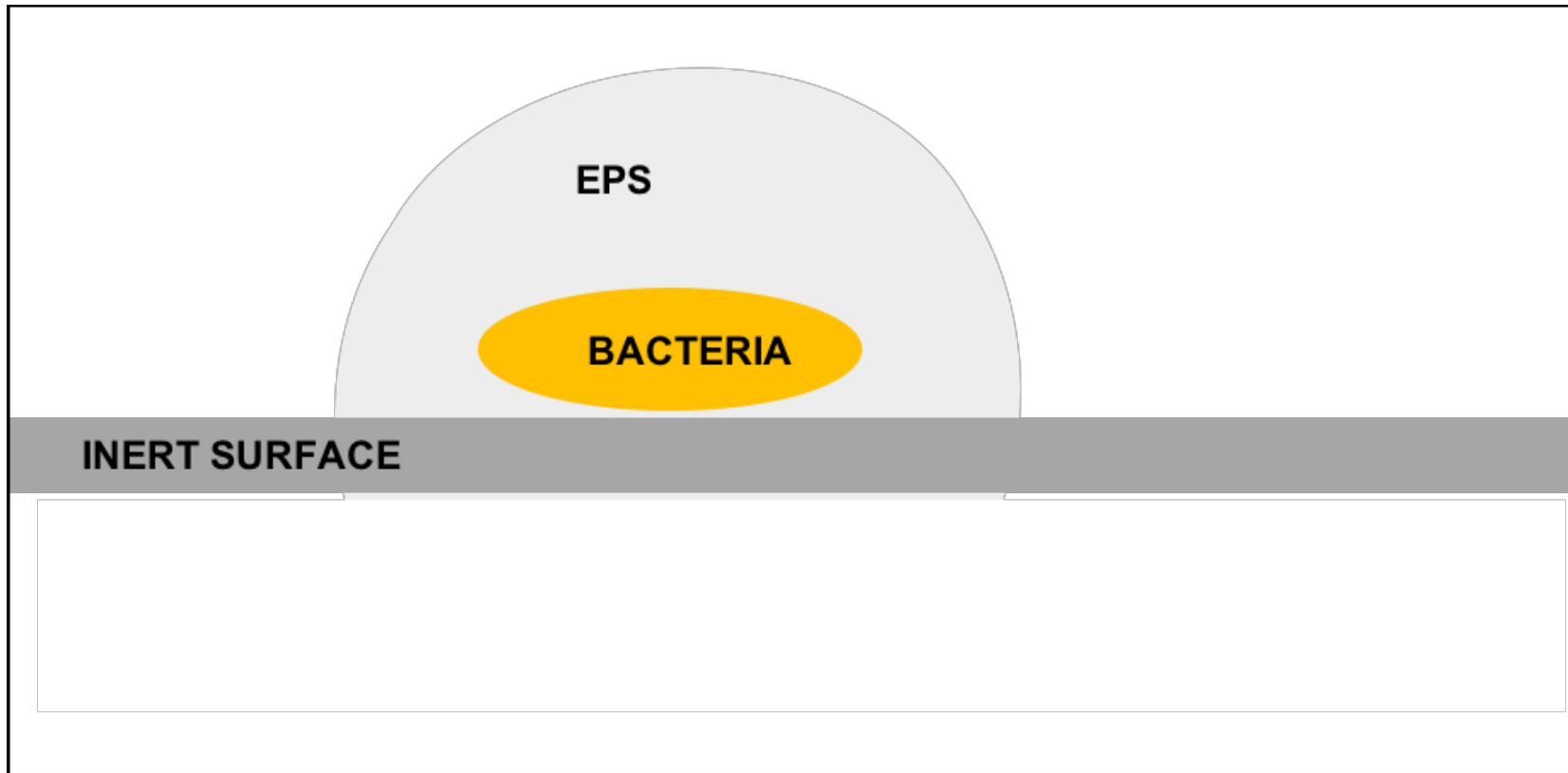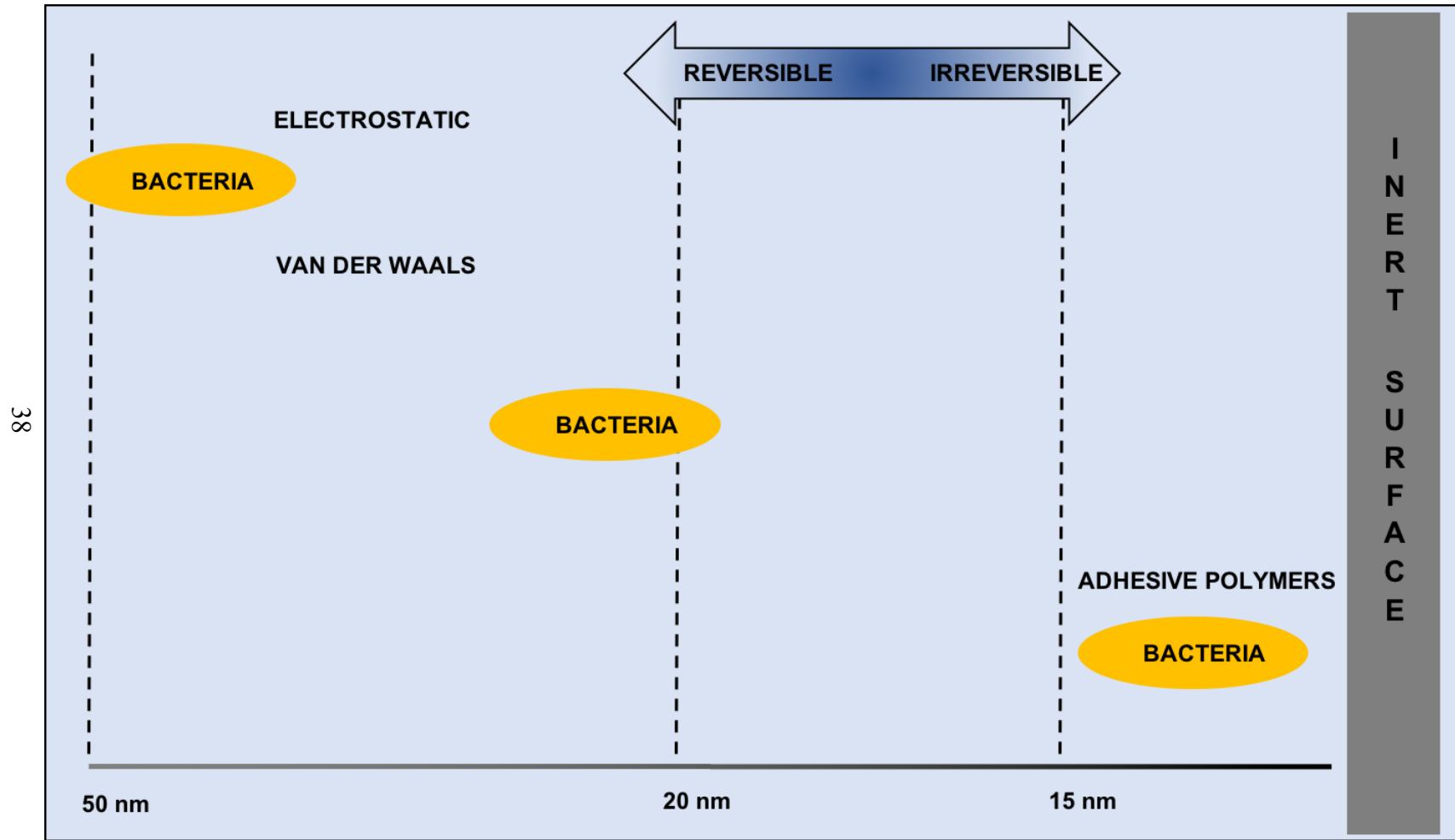
Figure 3C. Three-stage process of bacterial attachment. Adapted from Busscher and Weerkamp, (1987)

Originally attachment was thought to be due to the flagella and fimbriae. Since then, numerous studies have indicated the bacterial cells with or without flagella and fimbriae still attach at similar rates (McMeekin and Thomas, 1978; Noterman *et. al.*, 1980; Lillard, 1985; Selgas *et. al.*, 1993). However, more recent molecular and biochemical work on biofilms has indicated the presence of curli fibers, thin aggregative fibers, that are expressed in almost all *Salmonella* spp., *E. coli* (Bäumler *et. al.*, 1997; Collinson *et. al.*, 1996, Doran *et. al.*, 1993) and perhaps in other *Enterobacteriaceae*, such as *Shigella*, *Citrobacter*, and *Enterobacter* spp. (Doran *et. al.*, 1993). Curli fibers in *Salmonella* require the operons *csgDEFG* and *csgBAC* to be fully functional (Bäumler *et. al.*, 1997; Kim and Wei, 2009). Additionally, cellulose and curli production require the activation protein CsgD and expression typically occurs at temperatures below 86 ºF (30 ºC) (Kader *et. al.*, 2006; Kim and Wei, 2009). In an assessment of flagellated bacteria attaching to broiler skin, Notermans and Kampelmacher (1974) reported that optimal binding occurred at 20 ºC, further reinforcing the current data in that temperature plays a role in the regulation of CsgD protein expression.

Reduction of Microbial Load in the Processing Plant by Antimicrobials

There are several factors that contribute to the bacterial load of processed poultry carcasses, such as, level of contamination from live birds, numbers and genera of organisms introduced at pre-harvest, and the extent of contamination or cross-contamination during post harvest processing (Whyte *et. al.*, 2004). Aerobic bacterial levels of poultry entering the processing abattoir can be anywhere from mean $\log_{10}$ 6 to 9 CFU/mL or 4×10^8 to 4×10^{11} CFU/carcass and are mainly present on the feathers and skin (Kotula and Pandya, 1995; Lillard, 1989; 1990a). Broilers are first brought into the live hang department where birds are exposed to physical treatment hurdles to remove organic matter and feathers. Scalding water and feather

removal reduce the bacterial and organic matter significantly where mean bacterial counts of post pick carcasses are $\log_{10}$ 4.0 to 5.5 (James *et. al*., 1992; Lillard, 1989; Whyte *et. al.*, 2004 ; Brewer *et. al*., 1995). The reduction in microbial load by the physical processes of feather removal and hot water can be as much as two logs, as reported by Lillard (1989; 1990a).

Carcasses subsequently undergo evisceration processes where the microbial load is reduced again because of a washing affect (Carrasco *et. al.,* 2012). The evisceration process utilizes potable water to clean the equipment and carcasses during processing. The Inside Outside Bird Wash (IOBW) operates with pressure nozzles at 40 to 180 psi (Keener *et. al*., 2004) and can use up to 50 ppm of free chlorine to remove visible fecal contamination. Typical counts for carcasses post evisceration or pre-chill range from mean $\log_{10}$ 3.0 to 5.5 (James *et. al*., 1992; Lillard, 1989; Whyte *et. al.*, 2004; Brewer *et. al.*, 1995). The last step in slaughter is to cool the eviscerated carcasses. Bacterial counts post immersion chilling range in mean $\log_{10}$ from 2.5 to 3.7 CFU/mL (Mead and Thomas, 1973; Izat *et. al*., 1988; Lillard 1989; James *et. al*., 1992; Blank and Powell, 1995; Brewer *et. al*., 1995; Cason *et. al*., 1997; Bilgili *et. al*., 2002; Northcutt *et. al*., 2003; Handley *et. al.*, 2010). James *et.al.* (2006) reported a 2.5 $\log_{10}$ CFU/mL reduction for coliform and *E. coli* is possible with the addition of chlorine to an immersion chiller. Northcutt *et. al*. (2008) also collected microbial data where *E. coli*, coliform, and *Campylobacter* were reduced by 1.5, 1.5, and 2.0 $\log_{10}$ CFU/mL from pre-chill to post chill samples. Bacterial counts post air chilling were from mean $\log_{10}$ 3.4 to 5.5 (Barbut *et. al*., 2009; Clouser *et. al*., 1995; Whyte *et. al*., 2004; Salvat *et. al*., 1993). The difference of microbial numbers in air chilling and immersion chilling may be attributed to the washing effect of water, as Mead (2004) noted no modern air chilling system has the washing effect of immersion chilling.

The application methods for antimicrobials can be accomplished through dips or spray cabinets. Poultry processors can utilize antimicrobials from the USDA's Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Product list (2011) that has been approved for such an application. Loretz *et. al.* (2010), noted variation in directly comparing antibacterial activity between treatments and studies due to varying processing conditions, such as the mode of application (spraying, immersion, immersion chilling), the concentration, the application temperature, the exposure time, the point of application during processing, or the contamination level of the carcasses (Corry *et. al.*, 2007; Purnell *et. al.*, 2014). Lastly, the production floor layout may not allow for a properly sized dip tank required to achieve an adequate kill. For instance, immersion chillers with antimicrobials provide a high contact time in low temperature water with dwell times anywhere from 60 to 120 minutes; depending on the size of the chill tank, the carcass size, and processing line speed (Stopforth *et. al*, 2007). Conversely, spray-based systems would be easier to implement (retrofit) in an industrial situation, but it would need to be confirmed that the spray is coming in contact with the bird and ensure they are not clogged (Purnell *et. al.*, 2014). Either system has its benefits and short comings, but when choosing such interventions plant management must consider the efficiency and effectiveness of the treatment, the cost of the chemical, overall safety to employees and the environment.

Laboratory investigations commonly perform dipping trials of small inoculated meat samples in order to gain perspective on their antimicrobial potential (Millilo and Ricke 2010; Millilo *et. al.*, 2011; Cosansu and Ayhan, 2012; Alonso-Hernando *et al.* 2012, 2013). These studies offer a glimpse into how an antimicrobial may perform, but they need to be validated in the production facility to verify efficacy. In order to reduce the microbial load, it is paramount to utilize an intervention strategy and monitor its efficacy to meet the demands of both customer

and USDA regulatory compliance (Stopforth *et. al.*, 2007). Compliance with 9 CFR 417 requires the HACCP plan to be validated to ensure that the critical control points are operating as expected to reduce, control, and eliminate biological hazards (USDA-FSIS, 1996). When considering antimicrobial chemical interventions, the method of application and the antimicrobial compound type must also be considered. As for the antimicrobial compound, it is necessary to understand how the mechanism works in addition to the necessary operating parameters, such as time of contact, concentration and temperature (Stopforth *et. al.*, 2007; Purnell *et. al.,* 2014).

Common anti-microbial compounds used in poultry production

Chlorine

Chlorine has been a common antimicrobial for poultry processors in chiller water for years due to its broad-spectrum disinfecting property. (Milillo *et. al*., 2011; Ricke *et. al.,* 2005; Keener *et. al*., 2004). Typically, chlorine is added to the water system where it will react with water to form hypochlorous acid (HOCL), which acts as the antimicrobial agent (Hinton *et. al*., 2007; Keener *et. al*., 2004; Bailey *et. al*., 1986). The efficacy is dependent on contact time, the temperature, pH, chemical composition of water, and most importantly concentrations as free chlorine levels (Stopforth *et. al*, 2007; Tsai *et. al*., 1992). The USDA requires the use of 20 to 50 ppm chlorine in chiller water for the prevention of cross-contamination (Stopforth *et. al.,* 2007). However, the USDA's Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Product list (2011) (Table 1), has identified other chlorine compounds available for use, such as acidified sodium chlorite, calcium hypochlorite, chlorine gas, chlorine dioxide, sodium hypochlorite per USDA's specified parts per million and application purpose. For chlorine to be efficacious, the pH of water must remain in a range of 5.8 to 6.8 as nearly 100% of chlorine

added becomes hypochlorous acid (HOCL); at a pH of 8.5, only 8% of the chlorine added is converted to HOCL (Keener *et. al.*, 2004). Chlorine has shown to be lethal in low doses. Blaser *et. al.* (1986) presented data where as little as 0.1 mg/L (0.1 ppm) free available chlorine at pH 6.0 effectively reduced the *Campylobacter* population by 99%. However, chlorine binds readily to organic material and when added to a body of water free chlorine will bind until total chlorine demand has been met (Stopforth *et.al.*, 2007). After the total demand is achieved, then any free chlorine remaining will remain free to act as an antimicrobial (Tsai *et. al.*, 1992; Stopforth *et.al.*, 2007). Therefore, organic material from the skin of the bird, such as fats and dirt can bind to the free chlorine which would require more chlorine to be added. Therefore, it must be monitored closely in order to maintain effective chemical composition during slaughter.

The antimicrobial action of chlorine has been suggested to disrupt the transportation of extracellular nutrients (Campers and McFeters, 1979). Green and Stumpf (1946) noted that sulphydryl (-SH) groups for enzymes may be irreversibly oxidized, therefore destroying enzymatic activity. The high reaction rate at which active chlorine (OCl⁻) reacts with free thiol groups in cysteine residues is 3.0×10^7 M^{-1} s^{-1} at a pH of 7.4 (Imlay, 2003; Wang, 2009). Chlorine is also able to react with other amino acid side chains that enable it to easily oxidize protein residues exposed on the surface or transiently cross the cell membrane, thus leading to more oxidative damage (Pattison and Davies, 2001; Wang, 2009). In a genetic analysis of *E. coli* O157:H7 under oxidative stress conditions performed by Wang (2009), significant upregulation of the following genes and operons occurred: the multiple antibiotic resistance (*marRAB*) operon, the pathway for degradation of formaldehyde *frmRAB*, expression of the envelope stress response gene *spy*, the iron sulfur (Fe-S) cluster *isc* operon, the cysteine amino acid *cys* operons CND, JIH, PUWAM, and ZK. In addition, heat shock protein (Hsp) chaperone genes and two

biofilm inhibiting genes *ycfR* and *ybiM* were induced under oxidative stress caused by chlorine (Wang, 2009). Multiple cellular defense mechanisms are initiated by oxidative damage and requires the cell to expend ATP for the necessary repair of the cell. Therefore, chlorine is a popular choice as an antimicrobial due to its low cost and ability to destroy both spoilage and pathogenic bacteria (Lillard, 1979; Bailey *et. al.*, 1986; Hinton *et al.*, 2007; Keener *et. al.*, 2004).

There has been some drawbacks for chlorine even though it is extremely successful as an antimicrobial. Chlorine may have adverse reactions with organic compounds and amino acids present in all food, forming chlororganic compounds and chloramines. These compounds possess toxic properties including mutagenicity, teratogenicity, and carcinogenicity (Wei *et. al.*, 1985; Kuo *et. al*, 2000). Due to this potential issue, the European Union (EU) has prohibited chlorine and any other form of chemical decontamination (Mead, 2004). In response to concerns presented by the EU, a scientific panel was assembled to evaluate the concerns presented by the EU. During the analysis, various concentrations and time intervals of common poultry meat decontaminants were exposed to poultry meat and found to be of no safety concern so long as they were applied as prescribed by the manufacturer's (Anton *et. al.*, 2005). Since chlorine has been banned by the EU and preference changes by consumers to consume food with less or no additives, other solutions have been investigated (Corry *et. al.*, 2007). These changes in consumer trends had led to research on other antimicrobial hurdles besides chlorine, such as organic acids, steam, or hot water treatment.

Table 1. List of poultry chlorinated antimicrobials from the Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Products.

Antimicrobial	Trade Name or Product form	Concentration	Location of processing application
Acidified Sodium Chlorite	Sanova	500-1200 ppm in combination with any GRAS acid at a level to achieve a pH of 2.3 to 2.9 in accordance with 21 CFR 173.325.	Spray or dip
Acidified Sodium Chlorite	Sanova	50-150 ppm in combination with any GRAS acid at a level to achieve a pH of 2.3 to 2.9 in accordance with 21 CFR 173.325.	Pre-chiller or chiller
Calcium hypochlorite	Granular/ pellet form	50 ppm calculated as free available chlorine	Spray, dip, and chiller
Calcium hypochlorite	Granular/ pellet form	20 ppm calculated as free available chlorine. USDA has allowed the use of up to 50 ppm. 9 CFR 381.91	Re-work spray
Calcium hypochlorite	Granular/ pellet form	Not to exceed 5 ppm calculated as free available chlorine from the influent to the chiller	Red Water (poultry chiller water re-circulated through a heat exchanger to then re-enter into the chiller
Cetylpyridinium chloride (CPC)	Cecure	As an ambient temperature spray that must contain propylene glycol complying with 21 CFR 184.1666 in a concentration 1.5 times greater than that of CPC. Must also comply with 21 CFR 173.375. Applied before or after chilling, CPC cannot exceed 5 gallons per carcass provided that the system recaptures 99% of the solution and the concentration may not exceed 0.8% of carcass weight. Plus the application of CPC to the carcass must undergo washing either by a potable water spray or immersion chilling.	The surface of raw poultry carcasses or giblets before or after chilling

Table 1. (Cont.)

Antimicrobial	Trade Name or Product form	Concentration	Location of processing application
Chlorine gas	gas	50 ppm calculated as free available chlorine	Spray or chiller water influent
Chlorine gas	gas	5 ppm	Red water
Chlorine gas	gas	20 ppm calculated as free available chlorine. USDA has allowed the use of up to 50 ppm. 9 CFR 381.91	Reprocessing contaminated birds
Chlorine dioxide	gas	Not to exceed 3 ppm residual chlorine dioxide determined by Method 4500-ClO_2 E in the "Standard Methods for the Examination of Water and Wastewater", 18th ed., 1992, or an equivalent method and in accordance with 21 CFR 173.300	Water used in poultry processing
Sodium hypochlorite	solution	50 ppm calculated as free available chlorine	Spray, chiller water
Sodium hypochlorite	solution	5 ppm calculated as free available chlorine	Red water
Sodium hypochlorite	solution	20 ppm calculated as free available chlorine. USDA has allowed the use of up to 50 ppm. 9 CFR 381.91	Reprocessing contaminated poultry carcasses

Short Chain Fatty Acids

Several organic short chain fatty acids have been studied for application with the food industry and they include, acetic, benzoic, formic, citric, lactic, and propionic acid (Mulder *et. al.*, 1987; Izat *et. al.*, 1990; Dickens *et. al.*, 1994; Theron and Lues, 2007; Bauermeister *et. al.*, 2008). Additionally, they exist in a pure or buffered state containing 10 or less even-numbered carbons in their structural backbone (Theron and Lues, 2007). The application of organic acids or short chain fatty acids (SCFA) is best at a low or more acidic pH as the organic acid remains in an uncharged, un-dissociated state where the molecule is able to freely pass through the plasma membrane (Brul and Coot, 1999; Theron and Lues, 2007). The organic acid will continue to diffuse into the cell until equilibrium is achieved across the membrane in accordance with the pH gradient (Booth and Kroll, 1989; Brul and Coote, 1999). It is believed that the organic acids penetrate the lipid bi-layer of the cell membrane where the neutral pH of the cytoplasm promotes the organic acid to dissociate into the anions and protons (Eklund, 1983, 1985; Salmond *et. al.*, 1984; Cherrington *et. al.*,1990; Davidson, 2001; Ricke, 2003a). The un-dissociated organic acid is then thought to disrupt the membrane and its proteins involved in electron transport essential for ATP cycles, which then has the following cascade effect of inhibiting necessary metabolic reactions, causing an increased stress on intracellular homeostasis, and finally an accumulation of toxic anions (Brul and Coot, 1999; Ricke, 2003a; Theron and Lues, 2007).

In a study performed by Price-Carter *et al.* (2005), *Salmonella enterica* with mutations in polyphosphate kinase were able demonstrate an intracellular pH drop following exposure to the organic acids acetate and propionate by monitoring the inactivation of the acid and temperature sensitive enzyme MetA. It is suggested that polyphosphate kinase stimulates ATP-dependent proteolysis of ribosomal proteins during starvation to provide a source of amino acids (Kuroda

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et. al., 1999; 2000) and MetA is one of these proteins involved in protein synthesis, therefore making the enzyme an appropriate target to study (Price-Carter *et al.*, 2005). The ability of un-dissociated organic acid to react with or disrupt membrane integrity and membrane protein structure function, along with cytoplasmic proteins and DNA located within the cell (Davidson, 2001; Ricke, 2003a) allows for the accumulation of toxic anions and protons requiring the cell to rid itself of these to maintain homeostasis. Depending on the concentration of protons and anions present, the cell must rely on the buffering capacity of the cytoplasm, proton pumps, the glutamate decarboxylase system, acid tolerance response (ATR), proteins that repair or breakdown cell components, or alterations in the cell envelope (Theron and Lues, 2007; Alonso-Hernando *et. al.*, 2009). Therefore, the cell possesses mechanisms to remove these toxins from within the cell, but a bacterial cell only has limited ATP available to remove them. Purging excess protons requires ATP; once the cell has consumed all available ATP, then the cell's defense mechanism has been compromised due to depletion or exhaustion (Davidson, 2001; Ricke, 2003a). Compromising the integrity of the cell structure or depleting it of all available energy are the necessary targets required for processors to overcome microbial contamination leading to microbially safe food yet maintaining quality products.

Peroxy Acetic Acid

Peroxyacetic acid or peracetic acid (PAA) has also become more widely used due to its antimicrobial efficacy with chemical properties associated with an oxidizer. (Kitis, 2004; McDonnell and Russell, 1999; Block, 1991). PAA at equilibrium contains acetic acid, hydrogen peroxide, peroxy acetic acid, and water (Kitis, 2004; Block, 1991). The equilibrium state of PAA contains three disinfectants which act as a multi-hurdle within one solution. However, it should be noted that the disinfecting power of hydrogen peroxide is not equivalent to PAA, due to the

concentration present in the prepared mixture (Wagner *et. al.*, 2002; Kitis, 2004). PAA's antimicrobial action is the dual action from reactive oxygen as a peroxide within the organic acid molecule, acetic acid, and is considered an organic peroxide. Peroxides are high-energy-state compounds that are thermodynamically unstable which promote instability and combustion. (Block, 1991; Kitis, 2004).

Peracetic acid is believed to oxidize sulfhydryl (-SH) and sulfur bonds (S-S) in proteins, enzymes, and other metabolites with double bonds (Kitis, 2004; McDonnell and Russell, 1999). Therefore, PAA disrupts chemiosmosis function of lipoproteins in cytoplasmic membranes and transport mechanisms associated with cell walls (Kitis, 2004; Baldry and Fraser, 1988; Leaper, 1984) The ability to disrupt protein function has been attributed to its property as a sporicide and ovicide (Block, 1991). PAA, intracellularly, has been characterized as disrupting essential enzymes, vital biochemical pathways, active transport in organelle membrane pathways, interaction with DNA bases, and inactivation of catalase (Kitis, 2004; Fraser, 1984; Leaper, 1984; Tutumi *et. al.*, 1973; Block, 1991).

Spontaneous decomposition of PAA occurs between the pH range of 5.5 and 8.2, but the biocidal form of PAA is the undissociated acid form (Kitis, 2004; Gehr *et. al.*, 2002; Colgan and Gehr, 2001). Additionally, the pk_a is 8.2; in more alkaline conditions, greater than pH 9, the dissociated form of PAA exists in solution, thus a decreased antimicrobial activity (Kitis, 2004; Baldry and French, 1989; Sanchez-Ruiz *et. al.*, 1995; Tutumi *et. al.*, 1973). Evidence of pH affecting antimicrobial efficacy was reported by Sanchez-Ruiz (1995) where a 2 to 3 log reduction of coliforms was exhibited at a pH of 7 as opposed to 10 (Kitis, 2004). Additionally, Baldry and French (1989) reported PAA being more efficacious at neutral and or more acidic conditions (Kitis, 2004). Lastly, PAA has been found to effective as an antimicrobial at a wide

range of temperatures, but it was more efficacious as the temperature increased (Kitis, 2004; Stampi *et. al.*, 2001)

As one of the active compounds in PAA, hydrogen peroxide (HP) has broad spectrum against bacteria, bacterial spores, viruses, and yeast (McDonnell and Russell, 1999; Block, 1991). Hydrogen peroxide is decomposed by peroxidases and catalase, commonly found in Gram negative bacteria (McDonnell and Russell, 1999). Additionally, it produces –OH radicals that react readily with essential cell components, lipids, proteins, and DNA, especially those with double bonds or sulfhydryl groups. (McDonnell and Russell, 1999; Block, 1991).

Trisodium Phosphate

Trisodium phosphate (TSP) is also another antimicrobial available for use in poultry processing and has been reported as an effective carcass decontaminant (Ricke *et. al.*, 2005; Anton *et. al.*, 2005; Stopforth *et. al.*, 2007; Purnell *et. al.*, 2014). TSP is applied as a spray or dip and is an 8 to 12% aqueous solution with a pH value of approximately 12 (Ricke *et. al.*, 2005; Anton *et. al.*, 2005; Sofos *et. al.*, 2013). The high alkalinity (pH 12) of the prepared antimicrobial disrupts the cell membrane causing the cell to leak and it also acts as a detergent with its surfactant like properties (Anton *et. al.*, 2005). Ricke *et. al.* (2005) also reported that reactive hydroxyl radicals may provide residual antimicrobial activity. However, due to the treatment generating large volumes of phosphate, TSP can a problem for both the environment and effluent disposal (Ricke *et. al.*, 2005; Purnell *et. al.*, 2014).

Multiple Hurdle Effect

The multiple hurdle effect is the process of subjecting bacteria to constantly increasing harsh conditions that require an organism to repeatedly adapt to new environments where death occurs due to exhausting all a cell's resources. This strategy is best described as a series of

antimicrobial treatments that when employed alone would yield a limited reduction, whereas the series eliminates or significantly reduces the presence of pathogens on a food surface (Leistner and Gorris, 1995; Sirsat *et. al*., 2009). The slaughter process (Figure 1.0) includes multiple steps requiring either chemical or physical interventions that effect the viability of bacterial organisms, therefore is considered a multi-hurdle process. In order to demonstrate the effectiveness of poultry processing interventions, Stopforth *et. al*. (2007) validated the antimicrobial capacity of each dip and spray cabinet in multiple processing plants where each individual intervention resulted in statistically significant reductions of bacterial loads. During this investigation, the multiple spray cabinets demonstrated that the total system produced an effect similar to the multiple hurdle effect which reduced microbial populations much greater than each individual hurdle.

Current interventions available for meat processors to reduce pathogens and microbial load on carcasses include steam, hot water dips, and carcass rinses with chlorine, trisodium phosphate or organic acids (Hogue *et. al*., 1998, Corry *et. al*., 2007; Purnell *et. al*., 2014). Researchers and processors have begun to add multiple processing technologies within an individual processing step to increase effectiveness of the treatment (Millilo and Ricke, 2010; Millilo *et. al.,* 2011). The food industry has many antimicrobial applications available (Table 5) and combining any number of these may be possible provided they are synergistic in nature.

Hurdle technology allows individual interventions to be applied at either shorter time intervals or lower concentrations yet be more impactful (Leistner, 2000). Millilo *et. al.* (2011) demonstrated the effects of utilizing a mild heat, low pH, and select organic salts. The designer multihurdle treatment was very effective in reducing *Salmonella.* The same investigation examined a room temperature, 50°C, 55°C, and 60°C hot water dip with acidified organic acids

at either 1.25% or 2.5% sodium lactate, sodium acetate, and sodium propionate adjusted to a pH 4 and another group at pH 7. The dip was applied for one minute and they observed a greater than $\log_{10}$ 4 reduction using a 2.5% sodium propionate solution of pH 4 at 55°C. Millilo *et. al.* (2011) found that the heat increased membrane fluidity allowing the acid to penetrate more easily and the acidic condition of pH 4 created a significant pH gradient between the cytoplasm and the extracellular space.

The combination of heat, acid, and pH created a synergy that resulted in considerable microbial reductions. Millilo *et. al.*, (2011) reported that transcription of 203 genes was repressed and 150 genes were induced. Interestingly, those genes repressed were related to functions of heat shock response or molecular chaperones and those genes upregulated were related to cell attachment and/or mobility (Millilo *et. al.*, 2011). Such a demand on the microbial cell creates a considerable demand for the consumption of ATP. Typically, cells undergo a lag phase upon exposure to new environments or sublethal injury, this period allows the cell to adapt to the physiological elements (Swinnen *et. al.*, 2004). Employing similar synergistic antimicrobial hurdles are a promising strategy as it compromises multiple cellular components and functions rendering it too weak to survive.

Table 2. Hurdles used in food preservation (Leistner and Gorris, 1995)

Physical	Physiochemical	Microbially derived	Miscellaneous
High temperature, low temperature, ultraviolet radiation, ionizing radiation, electromagnetic energy (microwave, radiofrequency energy, oscillating magnetic field pulses and high electric field pulses), photodynamic inactivation, ultrahigh pressure, ultrasonication, packaging film, modified-atmosphere packaging, aseptic packaging, and food microstructure	Low water activity, low pH, low redox potential, salt, nitrite, nitrate, carbon dioxide, oxygen, ozone, organic acids, lactic acid, lactate, acetic acid, acetate, ascorbic acid, sulfite, smoking, phosphates, glucono-δ-lactone, phenols, chelators, surface treatment agents, ethanol, propylene glycol, Maillard reaction products, spices, herbs, lactoperoxidase, and lysozyme	Competitive microbiota, protective cultures, bacteriocins, and antibiotics	Monolaurin, free fatty acids, chitosan, and chlorine

Measuring the Efficacy of the Antimicrobial System

Antimicrobials commonly undergo evaluations by researchers and industry to better understand the efficacy and to investigate potentially new improvements for the current system. From a regulatory standpoint, monitoring is required per 9 CFR 381.94 (USDA-FSIS, 1996b) and validation of the hurdles in place verifies the HACCP Plan per 9 CFR 417 (USDA-FSIS, 1996a). Monitoring the intervention will ensure the optimization of the intervention strategy to effectively reduce the microbial load from live hang to post chill (Stopforth *et. al.*, 2007). A method to measure the intervention process is to perform bio-mapping.

Bio-mapping allows for the slaughter process interventions to be analyzed pre- and post-intervention throughout the whole plant (Figure 4A-C). The data obtained yields a visual map of the interventions being employed to produce a raw chicken carcass. The y-axis indicates the log 10 transformed microbial count and the x-axis is the sampling location. There are times in the process where the post testing location is the same as a pre- testing location and vice a versa, such as pre-OLR and post OLR. Post OLR is the same as Pre-chill in the examples (Figure 4A-C). The ideal state for the production system is a bar graph where each testing location has less CFU than the site before it. Figure 4A is an example of an ideal state; each processing hurdle is working within the designed parameters and is effectively reducing the bacterial load. Figure 4B is as effective as 4A in terms of the bacteria recovered at post chill. However, if the OLR had been operating within the designated limits, then the bacterial level could have been less than the results in 4A since the chiller reduced the microbial load by 4.5 logs. Figure 4C indicates that the chiller was not operating as expected due to the increase in bacteria recovered from the post chill samples. Even though testing is performed on individual hurdles, bio-mapping is a means of collectively analyzing the entire production system.

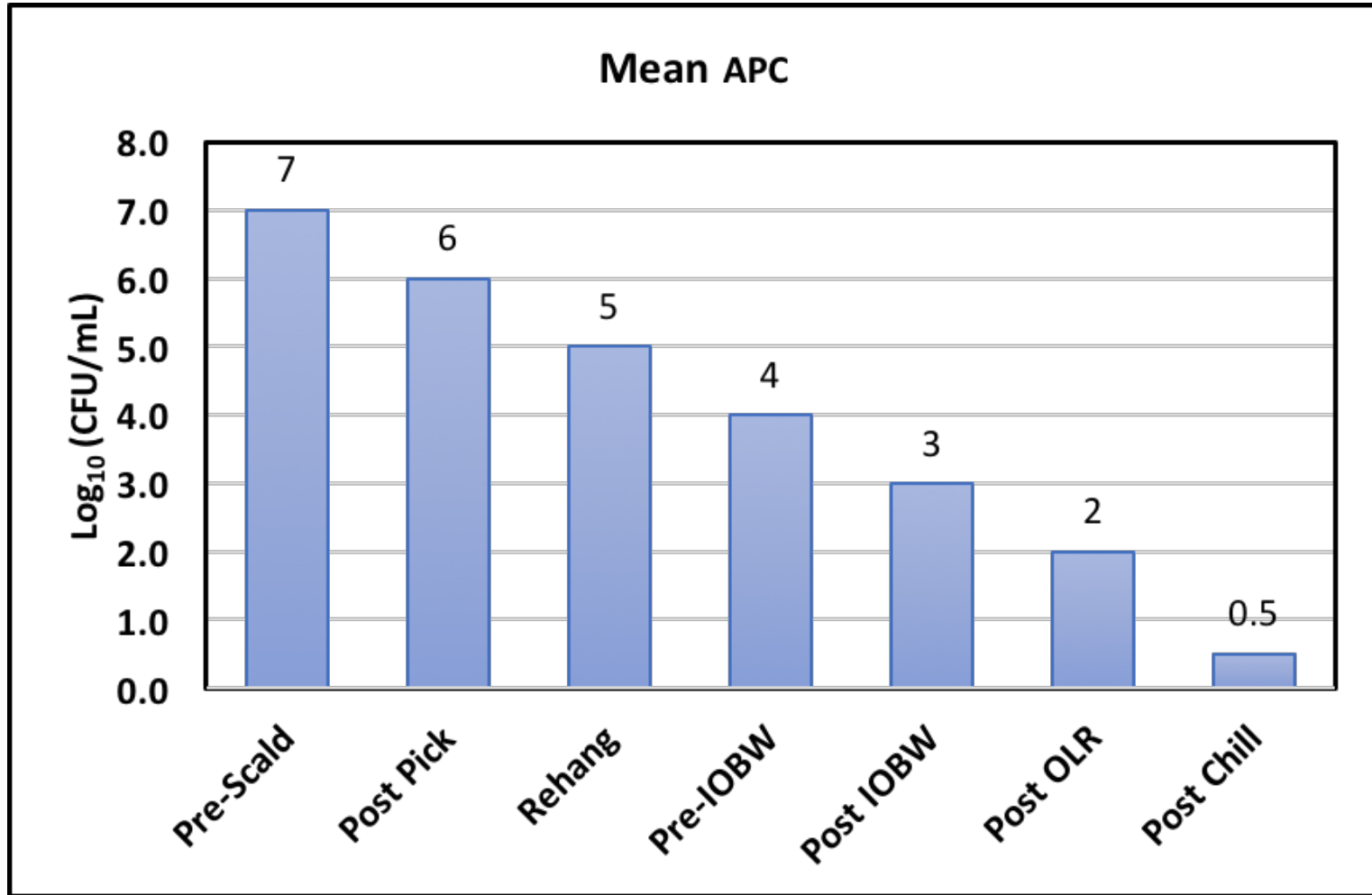Figure 4A. Ideal theoretical bio-map of the evisceration process. All hurdles are working as expected.

Figure 4B. Theoretical bio-map where the on-line reprocessing (OLR) intervention is not operating as expected.
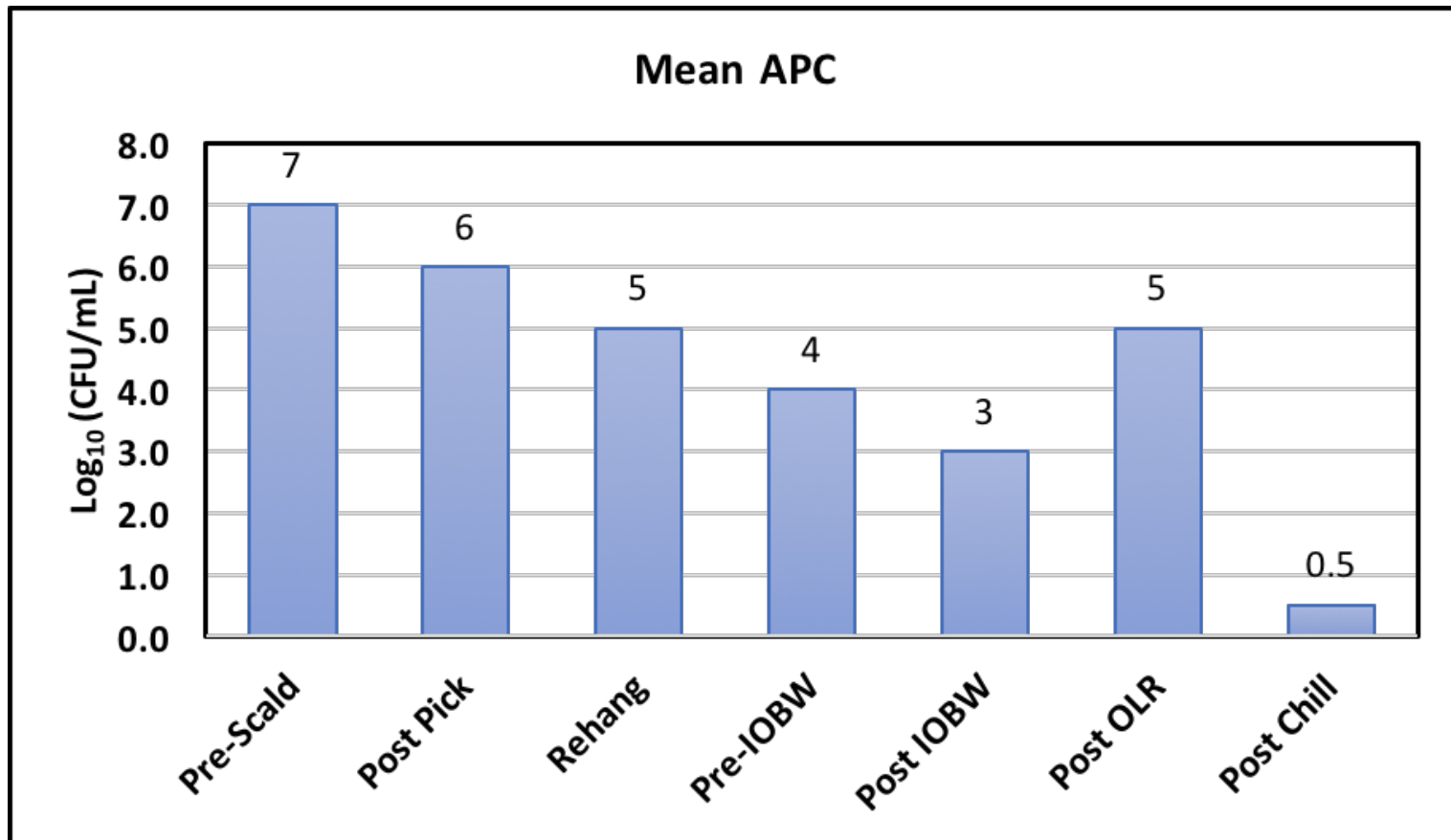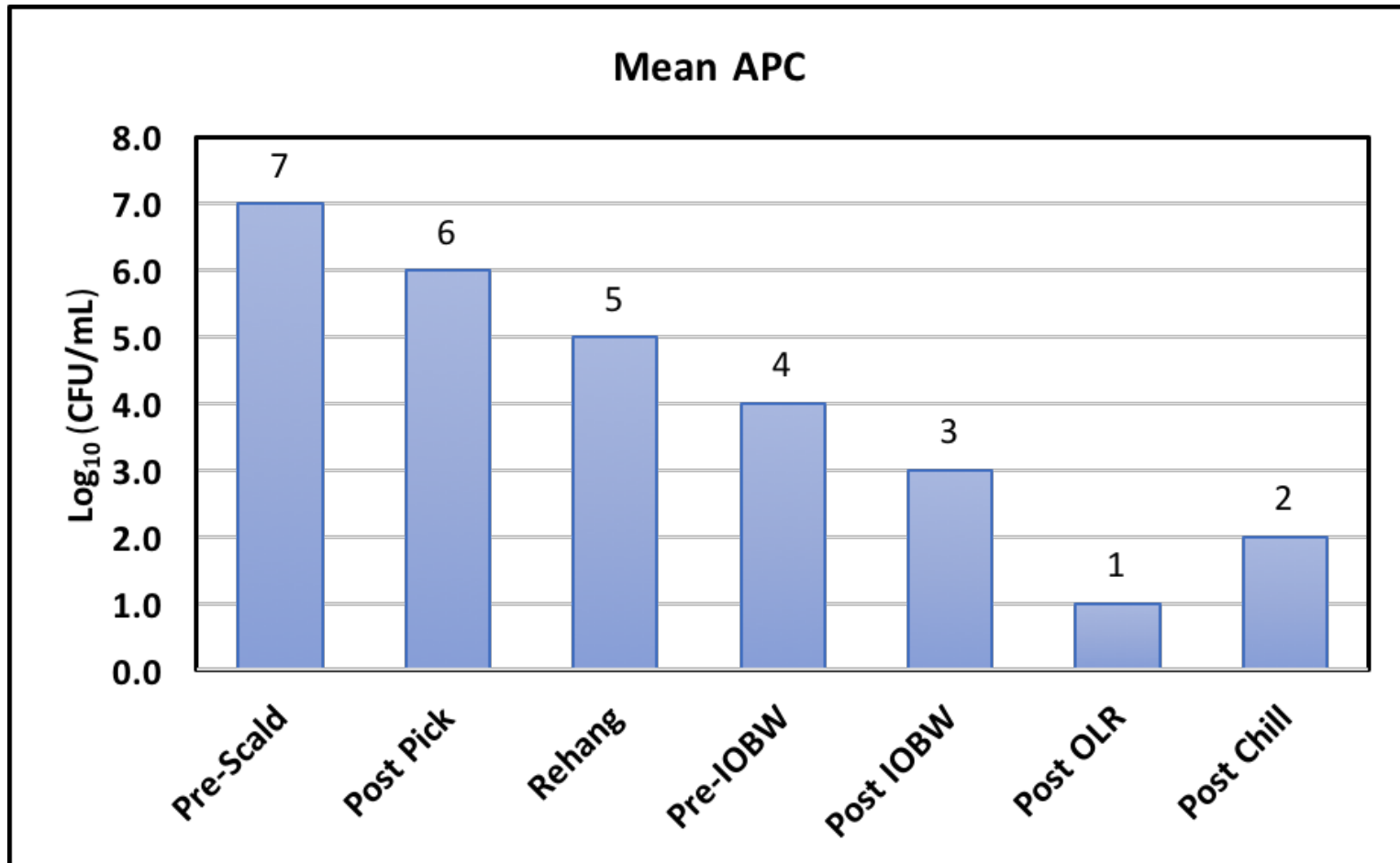
Figure 4C. Theoretical bio-map where the chiller is not operating as expected.

Microbial Ecology in Meat and Poultry

The microbiota of post chill broiler meat impact the quality and the shelf-life of the final product. Understanding the microorganisms present on post chill carcasses, provide insight into the good manufacturing practices and effectiveness of the processing abattoir multihurdle system. Refrigerated chicken meat is extremely susceptible to spoilage by psychrophilic microorganisms due the inherent physical properties of the harvested meat, such as its high water activity, neutral pH, and abundance of nutrients (Morales *et. al.,* 2016). Current data reflects evidence of bacteria present in post chill samples and how these organisms translocate will impact further processing of these carcasses and their quality for the consumer.

Typically, bacteria found on fresh processed poultry are mesophilic organisms that grow at moderate temperatures and some are considered pyschrotrophs, capable of metabolic activity in refrigerated (0.5 to 8.0°C) environments (Rao *et. al.*, 1998; Dickens *et. al.*, 2004; Forsythe, 2010; Blevins *et. al.*, 2017). They are considered spoilage organisms that are responsible for the breakdown of refrigerated meat products and their metabolites may produce off-odors as they consume the poultry product substrates available to them (Blevins *et. al,* 2017). Typical psychrotrophic spoilage genera found on aerobically stored, refrigerated raw meat have been isolated from feathers, feet of broilers, intervention water samples like chill tanks, and processing equipment (Russell *et. al.*, 1996).

The complex nature of spoilage can be directly linked to live animals and their processing environment. *Pseudomonas* spp. has been noted as the predominant microorganism of spoilage microbiota and impacts quality and shelf-life of raw poultry meat (Morales *et. al.,* 2016; Arnaut-Rollier *et. al.* 1999; Hanning *et. al.*, 2009) Researchers have commonly reported the following *Pseudomonas* spp as being recovered from raw spoiled poultry meat, *P. fragi, P. putida, P.*

fluorescens, P. lundensis, and *P. chlororaphis* (Arnaut-Rollier *et. al.*, 1999; Hanning *et. al.*, 2009; Hilbert *et. al.*, 2010; Morales *et. al.,* 2016). Nychas *et al*. (2008) concluded that under aerobic storage conditions three species of *Pseudomonas, P. fragi, P. fluorescens,* and *P. lundensis*, were responsible for slime and off-odors when they were present at $\log_{10}$ 7 to 8 CFU/ g. Other organisms have been reported as contributors of meat spoilage in addition to *Pseudomonas*, such as those stated by James *et. al.* (2006)*, Acinetobacter, Moraxella, Brochothrix thermosphacta, Aeromonas* spp., *Psychrobacter* spp. and Enterobacteriaceae. This is in agreement with others (Barnes and Thronley, 1966; Ellis, 2001; Charles *et. al*., 2006; Hilbert *et. al.*, 2010)

In more recent surveys of evisceration interventions utilizing microbiome 16S sequencing, Kim *et. al.* (2017) observed the following predominant bacteria Paenibacillaceae , *Bacillus*, *Gallibacterium*, *Lactobacillus*, Rikenellaceae, *Bacillales*, *Bacteroides*, Ruminococcaceae, *Pseudomonas*, *Veillonella*, and *Lentibacillus*. During a chill tank microbiome assessment Rothrock *et. al*. (2016) reported the presence of *Acinetobacter, Pseudomonas*, Pseudomondaceae, Enterobacteriaceae, Pasturellaceae, *Neisseria, Burkholderia, Streptococcus*, and *Lactobacillus*. Lastly, Oakley *et. al.,* (2013) characterized the microbiome of raw chicken parts exudate from retail packages and found *Pseudomonas* to have a 98% relative abundance, which other have commonly isolated (Barnes, 1972; Fung, 1987; Russell *et al*., 1996; Cox *et al*., 1998; Hinton *et al*., 2004).

In order to further improve bacterial reduction in the poultry slaughter system, it is necessary to better understand the remaining viable microbiota and how current intervention strategies may impact them. Discerning their origin and translocation to the final meat product enables further opportunity for greater process enhancements that will improve carcass and meat

product quality. Research has recovered various organisms using traditional techniques, but now next generation sequencing (NGS) may be able to close the gap in assessing carcass quality and origin of finished product microbiomes. More importantly, NGS may shed insight on to more ideal non-pathogenic indicator organisms that mirror the behavior of pathogens.

Table 3. Common spoilage bacteria genera for raw meat

Spoilage Organism	Authors
Pseudomonas	Barnes and Thronley, 1966; Barnes, 1972; Fung, 1987; Russell *et. al*., 1996; Cox *et. al.,* 1998; Arnaut-Rollier *et. al.* 1999; Ellis, 2001; Hinton et al., 2004; Charles *et al*., 2006; James *et. al.,* 2006; Nychas *et. al.*, 2008; Hanning *et. al.*, 2009; Hilbert *et. al.*, 2010; Oakley *et. al.*, 2013; Morales *et. al.,* 2016; Rothrock *et. al*. 2016; Kim *et. al*. (2017)
Pseudomondaceae	Rothrock *et. al*. 2016
Acinetobacter	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Brochothrix thermospacta	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Aeromonas	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Psychrobacter	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Enterobacteriaceae	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Paenibacillaceae	Kim *et. al*. 2017
Bacillus	Kim *et. al*. 2017
Gallibacterium	Kim *et. al*. 2017
Lactobacillus	Rothrock *et. al*. 2016; Kim *et. al*. 2017
Rikenellaceae	Kim *et. al*. 2017
Bacillales	Kim *et. al*. 2017)
Bacteroides	Kim *et. al*. 2017
Ruminococcaceae	Kim *et. al*. 2017
Veillonella	Kim *et. al*. 2017
Lentibacillus	Kim *et. al*. 2017
Moraxella	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Psychrobacter	Barnes and Thornley, 1966; Russell *et al*., 1996; Ellis, 2001; James *et. al.,* 2006; Charles *et al*., 2006; Hilbert *et. al.*, 2010; Rothrock *et. al*. 2016
Pasturellaceae	Rothrock *et. al*. 2016
Neisseria	Rothrock *et. al*. 2016
Burkholderia	Rothrock *et. al*. 2016
Streptococcus	Rothrock *et. al*. 2016

Indicator Organisms

The poultry industry has previously used indicator organisms for the assessment of food safety and quality attributes (Russel, 2000; Whyte *et. al.*, 2004; James *et. al.*, 2006; Kim *et. al.*, 2017). Indicator organisms have also been utilized as a means to measure good manufacturing practices (Russell, 2000). Researchers and processors have used the bacterial family *Enterobacteriaceae* to indicate the possible presence of *Salmonella* and *Escherichia* as these pathogens are in the same taxonomic family (Whyte *et. al.*, 2004). Others have used coliforms and *E.coli* as a means to detect fecal organisms on carcasses, post water immersion chilling (James *et. al.*, 2006). The USDA considers *E. coli* an indicator of fecal contamination and requires processors to use any quantitative method to analyze for *E. coli* (USDA-FSIS, 1996b). While it has been noted that meat products have a diverse population and focusing on one specific microorganism or group may not be all that informative (Rouger *et. al.*, 2017), microbiome-based research has suggested the opposite. As poultry carcasses approach the end of the slaughter process, the microbiota shifts to a less diverse population compared to live production (Kim *et. al.*, 2017; Oakley *et. al.*, 2013).

Ideally an indicator organism shares similar characteristics as the target organism, but is also in a much greater abundance than the organism of interest (Cason *et. al.*, 1997; Handley *et. al.*, 2015). In the case of poultry slaughter, *Salmonella* and *Campylobacter* are the target organisms of interest due to their ability to cause foodborne illness, but are in relatively low abundance in post chill samples. For example, broiler chicken whole carcass rinses are often cited as having an MPN value of 30 (Cox *et. al.*, 2011). Whereas evidence of the post chill microbial load for aerobic bacteria was mean $\log_{10}$ 2.5 to 3.7 CFU/mL (Mead and Thomas, 1973; Izat *et. al.*, 1988; Lillard 1989; James *et. al.*, 1992; Blank and Powell, 1995; Brewer *et. al.*, 1995;

Cason *et. al*., 1997; Bilgili *et. al*., 2002; Northcutt *et. al*., 2003; Handley *et. al.*, 2010). These organisms are more likely to be spoilage organisms and are present in much greater abundance than pathogens. Therefore, it is likely an organism present through the entire process of the farm to fork continuum may present itself as a candidate that could behave similarly to *Salmonella* and *Campylobacter*. Identification of an indicator organism will enable researchers and processors to assess the absolute efficacy of an antimicrobial as it would be deployed in a commercial processing environment (Cason *et. al.*, 1997; Handley *et. al.*, 2015; Blevins *et. al*., 2017).

Current Microbial Ecology Sampling

Detecting microbial shifts in the microbiota of poultry carcasses during interventions provides valuable data sets in relation to how an antimicrobial may target specific groups of bacteria (Sofos *et. al*., 2013; Giombelli and Gloria, 2014; Handley *et. al.,* 2015; Blevins *et. al.*, 2017). There are several nucleic acid based technologies (Table 4) that have been used over the years, but next generation sequencing (NGS) has begun replacing the other techniques for microbiome characterization (Oakley *et. al*., 2013, Rothrock *et. al.*, 2016; Koo *et. al.*, 2016; Kim *et. al*., 2017). The techniques used prior to NGS included Terminal Restriction Fragment Length Polymorphism (TRFLP), Automated Intergenic Spacer Analysis (ARISA), Denaturing / Temperature Gradient Gel Electrophoresis (D/TGGE) (Foster *et al*., 2012; Bokulich and Mills, 2012; Justé *et. al*., 2008) and a more recently developed method, denaturing high performance liquid chromatography (dHPLC) (Wagner *et. al.*, 2009). TRFLP and DGGE methods have been more commonly utilized in food matrices (Justé *et. al*., 2008) and dHPLC was a tool more recently developed and utilized for soil ecology and mutation analysis of single nucleotide polymorphisms. TRFLP was utilized for the differentiation of microbial populations by utilizing

a 5' fluorescently labeled primer to amplify the targeted gene (Justé *et. al.*, 2008; Bokulich and Mills, 2012). The amplified PCR product would be subjected to multiple terminal restriction enzymes and subsequently separated by capillary electrophoresis where the 5' fluorescently labeled terminal fragment was detected (Justé *et. al.*, 2008) and compared to an internally generated library (Bokulich and Mills, 2012). DGGE was a microbial characterization tool that exploited variable regions of 16S ribosomal RNA (rRNA) gene sequence fragments of the same length between 200 and 700 base pairs on an acrylamide gel with a denaturing gradient, formamide or urea (Muyzer *et. al.*, 1993; Muyzer and Smalla, 1998; Justé *et. al.*, 2008). A Guanine and Cytosine (GC) clamp was used to prevent 100% denaturation of double stranded DNA (dsDNA) and bands developed once the double stranded DNA separated with exception to the GC clamp (Justé *et. al.*, 2008; Bokulich and Mills, 2012).Lastly, dHPLC had utilized traditional ion-pair reversed phase HPLC systems with minor modifications to separate same length PCR products based on Guanine and Cytosine percent content (Wagner *et. al.*, 2009). Similar to other microbial profiling tools, a GC clamp was added to a variable region 16S rRNA gene primer (Barlaan *et. al.*, 2005). The sample was processed utilizing a nucleic acid column for HPLC; as the acetonitrile denaturing gradient increased, the partially denatured dsDNA strand released from the column generating a peak profile (Barlaan *et. al.*, 2005; Wagner *et. al.*, 2009)

More recent advancements in NGS have made the platform advantageous and appealing for researchers assessing the microbiota of various environments. Commercially available next-generation sequencer platforms have been developed for genomic and molecular research, such as Illumina MiSeq, Ion Torrent, and Pacific Biosciences (Quail *et. al,* 2012; Fichot and Norman, 2013; Escobar-Zapeda *et. al.*, 2015; Ricke *et. al.*, 2015, 2017; Comeau *et. al.*, 2017; Roumpeka *et. al.*, 2017). Application of 16S rRNA gene-based NGS has enabled researchers to census and

generate microbial profiles of complex ecosystems and environmental microbiomes (Ricke *et. al.*, 2017). Such an example would be the assessment of the farm to fork continuum in poultry production performed by Oakley *et. al.* (2013). They utilized high-throughput sequencing (HTS) to characterize the microbiome of chicken samples that included fecal, litter, carcass rinsates, and carcass weeps. Rothrock *et. al.* (2016) generated a microbial profile of both scalder and chiller tank waters from a commercial poultry processing plant over 3 days. Lastly, Kim *et. al.* (2017) reported the microbiota of whole carcasses undergoing the evisceration process. These studies identify discernible shifts in the microbiota present over a time and space continuum from live production to post evisceration.

Metagenomics is a powerful tool for the exploration of microbiomes and their dynamic communities, but processing the data can be difficult (Foster *et. al*., 2012). Typically, the 16S rRNA gene is the target sequence because all bacteria and acrchaea contain conserved genomic sequence regions and variable regions (V1-V9) specific to taxanomic groups (Robinson *et. al.*, 2016; Amato, 2017; Ricke *et. al.*, 2017). In some cases, it is more optimal to sequence multiple regions or a single region depending on the project objective; the more common choice is either the V1 to V3 region or the V4 region (Robinson *et. al.*, 2016; Amato, 2017; Ricke *et. al.*, 2017). There is also a limit to how many base reads can be used in the target, commercially available HTS like Illumina use base read lengths of 100 to 350 base pair (Robinson *et. al.*, 2016; Amato, 2017; Ricke *et. al.*, 2017). After sequencing, the raw data will have to be converted into digital data (Ricke *et. al.*, 2017). NGS raw data conversion uses databases with DNA sequences of organisms uploaded and the DNA matches are only as good as the genomes uploaded in the database (Justé *et. al*., 2008). Therefore, using a well-managed and curated database is best as they will minimize the addition of poorly sequenced microbial genomes (Foster *et. al*., 2012).

Some more commonly used databases are Greengenes, Silva, and the Ribosomal database project (Bokulich and Mills, 2012).

Current analysis of metagenomic data is best described by alpha and beta diversity as they provide insight into the diversity of the population and the relatedness of the organisms associated with the microbiome (Kim *et. al.*, 2017; Rothrock *et. al*., 2016; Ricke *et. al.*, 2017). Alpha diversity assesses the taxonomic complexity of the microbial community by providing the total number of genetically distinct operational taxonomic units (OTUs) within a sample (Ricke *et. al.*, 2017). Statistical tests commonly utilized to report alpha diversity are as follows: they measure the richness (Chao 1), evenness (Equitability), and diversity (Shannon) (Rothrock *et. al*., 2016). Rarefaction curves depict the above mentioned statistical test (Kim *et. al.* 2017). Beta diversity has best been described "accounting for the shared taxa among the sample microbial population versus missing taxa among these samples" (Ricke *et. al.*, 2017). The comparison between samples can be measured in terms of distance by comparing the presence or absence of OTUs and/or their abundance (Robinson *et. al.*, 2016;). As for beta diversity statistical analyses, weighted and/or unweighted, three-dimensional principal coordinated analysis (PCoA) UniFrac plots exhibit the relative abundance of OTUs among samples and their respective phylogenetic distances between the other samples. Commonly used bioinformatics program pipelines are mother and QIIME (Schloss *et. al.,* 2009; Schloss, 2010; Caporaso *et. al*., 2010; Gonzalez and Knight, 2012; Huse *et. al*., 2014; Nilakanta *et. al.,* 2014; Ricke *et. al.*, 2017).

Table 4: Microbial profiling tools in analyzing a microbiome

	DGGE	TRFLP	dHPLC	NGS
Nucleic acid technique (requires PCR)	x	x	x	x
Restriction enzyme usage		x		
Phylogenetic variation	x	x	x	x
Point mutation detection			x	
Quantitative		Pseudo		Pseudo
Sequence length (base pairs)	200-700	Random	200-1400	100-600
Throughput		x	x	x

Conclusions

The poultry slaughter process is a very complex network as the broiler traverses the farm to fork continuum. Evidence strongly supports the impact of horizontal transmission of microorganisms throughout live production and evisceration stages. It is imperative for food processors to develop and implement strategies to reduce microbial loads for reasons of food safety and consumer preference. In order to develop more effective antimicrobial hurdles, the employment of non-pathogenic indicator organisms that behave similar to pathogens are necessary. In the pursuit of establishing a suitable indicator organism, nucleic acid based research tools such as HTS of 16S rRNA genes have begun to provide new insight in regards to the microbial characterization of poultry throughout live production and evisceration (Oakley *et. al.*, 2013; Handley *et. al.* 2015; Rothrock *et. al.*, 2016; Kim *et. al.,*2017; Blevins *et. al.* 2017). Additionally, NGS is more than capable of detecting discernable shifts in the microbiota of poultry undergoing stages in mechanical evisceration and in the processing abattoir environment (Rothrock *et. al.*, 2016; Kim *et. al.,*2017) As demonstrated, NGS is the future of molecular biology (Oakley *et. al.*, 2013; Rothrock *et. al.*, 2016; Kim *et. al.,*2017) and as more data of the poultry microbiome increases so will the advancements in hurdle technology and food safety.

Premise for study

NGS has recently been employed as a tool to characterize whole carcasses in the poultry production system. This study will utilize microbiome-based 16S sequencing in conjunction with current quantitative plating techniques to profile the microbiota of chicken carcasses and determine the efficacy of the multi hurdle interventions in the poultry processing system.

Commercially processed broilers will be sampled from 3 different plants and at the locations with a star in Figure 2. Whole bird carcass rinses will be aseptically collected and

aliquot plated for quantitative analysis, validating the multi hurdle process. Another aliquot will

have the DNA extracted for metagenomic analysis. The characterized microbiota will be used to

validate the multi hurdle interventions employed in evisceration. Additionally, profiling the

microbiome may identify potential indicator organisms that could be used in the future

assessments of new antimicrobial interventions within a slaughter facility.

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Chapter 3

Microbiome Profiles of Commercial Broilers Through Evisceration and Immersion Chilling During Poultry Slaughter and the Identification of Potential Indicator Microorganisms

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Abstract

Commercial poultry abattoirs were evaluated to determine the efficacy of the multi-hurdle antimicrobial strategy employed to reduce the microbial load present on incoming broilers from the farm. As next generation sequencing (NGS) has been recently employed to characterize the poultry production system, this study utilized 16S High throughput sequencing (HTS) and quantitative plating data to profile the microbiota of chicken carcasses and determine the efficacy of the multi-hurdle antimicrobial system. Aerobic plate count (APC) and *Enterobacteriaceae* (EB) microbial counts were quantified from whole bird carcass rinsates (WBCR). The remaining rinsates underwent microbiome analysis using 16S rRNA gene fragments on an Illumina MiSeq and were analyzed by Quantitative Insights into Microbial Ecology (QIIME). The key stages of processing were determined to be at rehang, pre-chill, and post chill as per the *Salmonella* Reduction Regulation (75 Fed. Reg. 27288-27294). The APC microbial data from rehang, pre-chill, and post chill were mean log 4.63 CFU/mL, 3.21 CFU/mL, and 0.89 CFU/mL and EB counts were mean log 2.99 CFU/mL, 1.95 CFU/mL, and 0.35 CFU/mL. Next generation sequencing of WBCR identified 222 Operational Taxonomic Units' (OTU's) of which only 23 OTU's or 10% of the population was recovered post chill. Microbiome data suggested a high relative abundance of *Pseudomonas* at post chill. Additionally, *Pseudomonas, Enterobacteriaceae*, and *Weeksellaceae Chryseobacterium* have been identified as potential indicator organisms having been isolated from all processing abattoirs and sampling locations. This study provides insight into the microbiota of commercial broilers during poultry processing.

Introduction

The meat processing industry is subject to many regulatory requirements due to the association of foodborne illness outbreaks in which *Salmonella* spp. has been been the etiological agent in an estimated 1.0 million food borne illness cases (Scallan *et. al*., 2011; 75 Fed. Reg. 27288-27294). Regulatory requirements established in 1996, set forth by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS), required broiler processors to implement both a Hazard Analysis Critical Control Point System (HACCP) and to comply with performance standards for *Salmonella* spp. and *Escherichia coli* Biotype I (FSIS, 1996 a,b,c). In 2010, the USDA-FSIS introduced modifications to the regulation that both updated existing performance standards and added *Campylobacter* spp. performance standards for broilers (75 Fed. Reg. 27288-27294). Within the Code of Federal Regulations, 9 CFR 381.94 (USDA-FSIS, 1996b), poultry abattoirs are to test carcasses to demonstrate process control. Additionally, the HACCP plan must be validated annually per 9 CFR 417 (USDA-FSIS, 1996a) and interventions are apart of the HACCP plan.

Aside from the regulatory requirments, monitoring the microbial intervention will ensure optimal performance in reducing the bacterial load from live hang to post chill (Stopforth *et. al.*, 2007). Broilers brought into the slaughterhouse have been recorded as having aerobic bacterial levels ranging from mean $\log_{10}$ 6 to 9 CFU/mL or 4×10^8 to 4×10^{11} CFU/carcass (Kotula and Pandya, 1995; Lillard, 1989; 1990). In order to reduce the microbial load effectively, research efforts have focused on the reduction and elimination of both pathogenic and spoilage bacteria (Kim *et. al.*, 2017; Purnell *et. al.*, 2014; Millilo *et. al.*, 2011; Bauermeister *et. al.*, 2008; Northcutt *et. al.*, 2008; Stopforth *et. al.*, 2007; Mulder et al., 1987; Izat et al., 1990; Dickens et al., 1994; Yang et al., 1998; Lillard, 1994; Doyle and Waldroup, 1996). Therefore,

antimicrobials commonly undergo evaluations by researchers, both academic and industry, to investigate the efficacy and for improvements to the current system. A method to measure the intervention process is to perform bio-mapping.

Bio-mapping measures the microbial recovery pre- and post intervention for the whole process. Thus, a systematic analysis of each individual hurdle comprising the whole system. This map will effectively reveal where intervention strategies are successful or failing. In order to measure the effectiveness of commercial intervention strategies against potential pathogens, the employment of indicator organism can prove useful (Russel, 2000; Whyte *et. al.*, 2004; James *et. al.*, 2006; Handley *et. al.*, 2015; Kim *et. al.*, 2017). For instance, Enterobacteriaceae is a family of bacteria that contains pathogens such *E. coli* O157:H7 and *Salmonella* spp. (Whyte *et. al.*, 2004). An indicator organism would ideally be a non-pathogenic microorganism that behaves similarly to the environmental conditions as a target human pathogen and the population present in large enough quantities to be detected using cost effective microbiological techniques.

Carcasses entering the abattoir yield high levels of bacteria capable of degrading the product quality and/or causing human pathogenesis (Kotula and Pandya, 1995; Lillard, 1989; 1990; Stopforth *et. al.*, 2007). The identified microbiota present through various stages of food processing should enable researchers and industry experts to better develop product and intervention strategies (Hunter *et. al.*, 2009; Stern *et. al.*, 2001; Solow, 1993). The bacterial populations that are present can be indicative of contamination or it may be inherent to the product. Employing next generation sequencing tools, such as 16s RNA gene based microbiome sequencing could allow researchers to gain further insight into the microbial populations present through various niches in processing.

In this study, 16S high throughput sequencing (HTS) was utilized to establish a typical microbiome of commercially processed broilers. Furthermore, establishing next generation sequencing as an applicable tool, in conjunction with currently available plating techniques, to validate and measure the reduction in microorganisms by the antimicrobial multi-hurdle system of commercial processors. Lastly, this study evaluated the microbiome profile to identify potential indicator organisms that could benefit the broiler industry during bio-mapping.

Materials and Methods

Sample Collection

Whole chicken carcass rinsates were collected from three commercial broiler abattoirs. The birds were aseptically removed from the production line shackles during 1st shift production; each location had been processing for a minimum of 3 hours prior to sampling. A total of 30 rinsates were collected at each slaughter facility and each facility had 3 sampling points (Figure 1) defined as rehang, pre-chill, or post chill. In all, 90 carcasses were aseptically collected from the processing line and rinsed in pre-chilled 400 mL Butterfield's Phosphate diluent as prescribed in the FSIS-USDA Microbiological Laboratory Guidebook (MLG) (2017). The rinsates was placed back into the original Butterfield's Phosphate diluent container with screw lids sealed. They were placed on ice for transport and returned to the testing lab for analysis. Upon arrival the samples were placed into the refrigerator.

Bacterial enumeration

All samples were plated as described by the USDA MLG Chapters 3 and 41.5 on following media: 3M Aerobic Plate Count (APC) PetriFilm and 3M *Enterobacteriaceae* PetriFilm (3M Microbiology, St. Paul, MN, USA). Prior to enumeration, samples were re-suspended in their respective jars by shaking vigorously and subsequently performing 1:10 serial

dilutions in Butterfield's Diluent (BF's) (Edge Biologicals, Inc., Memphis, TN, USA). One

milliliter aliquots were directly plated from the sample and dilution tubes onto the corresponding

PetriFilm. PetriFilm plates were incubated at 35°C in aerobic conditions per the manufacturer's

directions. Samples were incubated per the manufacturer's directions and colonies were

enumerated and calculated as total colony forming units (CFU) per mL for each dilution.

DNA Extraction

A 50 mL subsample of the original 400 mL WBCR was transferred into a sterile 60 mL

conical tube. The conical tubes were spun down using an Thermo Scientific Sorvall Lynx 6000

(Langenselbold, Germany) at 8,000 x g for 15 minutes. The supernatants were poured off and the

pellets were subsequently re-suspended in 2 mL of phosphate buffered saline (PBS). DNA

extractions were performed using a Fisher Scientific AccuSpin Micro 17 (Langenselbold,

Germany) and a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) with modifications

to increase DNA yield (Park *et. al.,* 2014, 2016). The specific modifications were performed

prior to the QIAamp Stool Mini Kit (Qiagen, Valencia, CA, USA) and included the addition of

0.7 mm garnet beads (MO BIO Laboratories Inc., Carlsbad, CA, USA) and vortexing for 1

minute. The samples were centrifuged and the supernatant was transferred to a fresh 2 mL

microcentrifuge tube containing 0.1 mm glass beads (MO BIO Laboratories Inc., Carlsbad, CA,

USA). Those tubes underwent horizontal vortexing for 10 min. and then incubated in a 95°C heat

block for 6 min. (Park *et. al.,* 2014). QIAamp DNA Stool Mini Kit was performed as prescribed

by the manufacturer. All samples were analyzed on a Qubit® 2.0 Fluorometer (Life Technology,

Carlsbad, CA, USA) to determine the isolated DNA concentration followed by dilution to 10

ng/µL.

Library Preparation

The isolated DNA aliquots were utilized to construct a sequencing library that targeted the V4 region of 16S rRNA as suggested by Kozich (2013). Individual DNA samples were amplified with dual-index primers through PCR and amplicons were normalized using the SequalPrep™ Normalization Kit (Life Technology) per the manufacturer's recommendation. Each sample contained unique barcode sequences, at both the front and end of the PCR amplicon, to distinguish each sample sequence in a pooled library. The pooled library contained a 5 µL aliquot of each normalized sample and was used for further assays. Once pooled, the library concentration and the exact DNA product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) through quantitative PCR (qPCR, Eppendorf, Westbury, NY, USA) assay and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively. Based on the qPCR and bioanalyzer results, the pooled library was subsequently diluted to 4 nM prior to sequencing.

Sequencing via an Illumina MiSeq Platform 157

A pooled library (20 nM) and a PhiX control v3 (20 nM) (Illumina) were mixed with 0.2 N fresh NaOH and HT1 buffer (Illumina) to produce the final concentration of 12 pM's each. The resulting library was mixed with the PhiX control v3 (5%, v/v) (Illumina) and 600 uL loaded on a MiSeq® v2 (500 cycle) Reagent cartridge for sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® website.

Sequencing Data Processing

Both demultiplexed R1 and R2 sequencing read (approximately 250 bp in length) files were acquired from the Illumina BaseSpace® website and data processing was performed using

the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version 1.9.1) (Park *et. al.*, 2016; Caporaso *et. al.*, 2010). Clustered sequences were used to assemble Operational Taxonomic Units (OTUs) tables with 93.93% identity and classified into the respective taxonomical level from domain to genus based on the Greengenes 16s rRNA gene database. Within the QIIME 1.9.1 package, both alpha diversity and beta diversity data were obtained. Alpha diversity data included rarefaction curves for OTUs and Chao1, while beta diversity data included weighted and unweighted UniFrac distances to characterize the microbial population.

Statistical Analysis

All bacterial counts were log 10 transformed, prior to analyzing the mean and standard deviation of each individual plant. A One-way Analysis of Variance (ANOVA) or Tukey's Honest Significant Difference Test was performed using JMP® (version 13.1.0). Microbiome alpha and beta diversity were calculated by using QIIME pipeline (version 1.9.1). Additionally, quality metrics from the Illumina Mi-seq runs were obtained from Illumina BaseSpace® website.

Results

Quantitative Bio-mapping Results

The log means of individual and all evisceration microbial data for both APC and *Enterobacteriaceae* are presented in Table 1. The microbial reduction from rehang to post chill did have a statistically significant reduction, *p*-value <0.001, as indicated by Table 2. The APC all plant mean log CFU/mL bacterial counts for rehang, pre-chill, and post chill were 4.63, 3.15, and 0.81, respectively; *Enterobacteriaceae* was 2.99, 1.79 and 0.12 respectively. In terms of bacterial load per carcass at rehang, pre-chill, and post chill, APC was 17,063,180 CFU/carcass, 565,015 CFU/carcass, 2,582 CFU/carcass, respectively; *Enterobacteriaceae* was 390,894 CFU/carcasss, 24,663 CFU/carcass, and 527 CFU/carcass, respectively. This data was utilized to

build the bio-map in Figure 2. The reduction from rehang to post chill for APC was 3.82 log

CFU/mL and *Enterobacteriaceae* was 2.86 log CFU/mL. Each step reduced the microbial

populations significantly and Figure 2 illustrates the reduction throughout the evisceration

process. In summary, bacterial counts continued to drop significantly from rehang to post chill

which yielded a negative slope, indicative of a processing system in control.

Taxonomic Summary

The microbiome data suggests that 95.01% of the organisms present were identified as

organisms from the phyla Bacteroides, Firmicutes, Proteobacteria and Actinobacteria. However,

the most abundant phyla, as noted in Figure 3, was Proteobacteria. Proteobacteria represented

48.0% of all genomes recovered, followed by Firmicutes with 31.7%, and Bacteroidetes with

11.3%. During the genome analysis of all the rinsates collected at the genus level, a total of 222

OTU's were identified and only 23 OTU's or 9.65% was recovered after post chill.

Since one objective was to investigate non-pathogenic indicator candidate organisms, the

ideal organism would be present at rehang, pre-chill, and post chill. Therefore, the genera were

first filtered by those observed in the post chill samples only. Therefore, Table 3 contains a list of

all genera recovered at all three post chill abattoirs. The list of organisms was further filtered by

requiring all organisms to be present in rehang, pre-chill, and post chill samples. Therefore,

Figure 4 indicates genera identified during all sampling stages and abattoirs for a total of 7

OTU's at the taxonomic level Family or Genus. The two taxonomic groups with the highest

relative abundance were *Pseudomonas* and *Enterobacteriaceae*. The post chill relative

abundance of *Pseudomonas* and *Enterobacteriaceae* was 83.5% and 2.2%, respectively.

Identified genera with a relative abundance >1.0% were analyzed at the species level (Table 4).

Few species at post chill were identified (Table 4) and those identified were <1.0%. The OTU's

most abundant were closely related to *Pseudomonas, Enterobacteriaceae,* and *Chryseobacterium* with a relative abundance of 94.8%, 2.2% and 1.13%.

QIIME Sequencing Metrics

During sequencing, 18,879,978 reads were generated and 17,730,162 of those reads passed filtering. Therefore, 93.93 ±0.53% of the sequence clusters passed filtration with an error rate of 1.75 percent. Additionally, BaseSpace reported 82.1% of base calls having a Q30 score or better; a quality metric indicating that 1 in 1000 base calls have a possible error. The summarized Illumina Mi-Seq read lengths and Shannon Diversity values obtained from QIIME are identified in Table 3. The standard deviations associated with Shannon diversity scores were obtained using JMP. As expected, the samples exhibited a more diverse population in the less processed rehang rinsates and as the carcasses were further processed they become less diverse. Additional alpha diversity results are from Chao1 and OTU's rarefaction curves presented in Figures 5 and 6. Both Figures 5 and 6 indicate the read lengths and the number of organisms' present for the associated sample location. These curves indicate that the diversity within the sample were higher during rehang and became less diverse by the end of post chill. The loss in community richness should be expected as the carcasses are undergoing cleaning steps and does resemble the finding obtained in the bio-map.

Beta diversity principle coordinate analyses, Figure 7, depicted the relatedness of identified OTU's between samples. Both weighted and unweighted UniFrac plots (Figure 7) were generated for plants 1, 2 and 3. The weighted PCoA UniFrac plot quantitativly measured the relative abundance of OTU's among a group. The unweighted PCoA UniFrac plot was a qualitative representation of phylogenetic distance based on the presence/absence of OTU's among samples in a group. Initial analysis of the PCoA plots for all organisms present indicated

less genetic diversity among the total population of young broilers (Figure 7E). As the birds increased in age the population grew in genetic diversity (Figure 7A). However, the inverse was true for the PCoA plots generated for *Pseudomonas* (Figure 8). Rather, the PCoA plots in Figure 8 indicate that broilers with an older slaughter age had greater similarity in genetic diversity for the population of *Pseudomonas* spp. Since *Pseudomanas* spp. had the highest relative abundance in all samples and locations collected, Figure 8 depicts weighted and unweighted PCoA plots generated for *Pseudomonas* spp. only. Figure 8 A, C, and E are the weighted PCoA plots for Plants 1, 2, and 3. These figures depict shifts in the relative abundance in *Pseudomonas* spp. as the birds increase in slaughter age, where Plant 1 (Fig. 8A) is the oldest and Plant 3 (Fig. 8E) is the youngest. As for the unweighted PCoA plots, Figure 8 B, D, and F, indicate a greater phylogenetic difference for *Pseudomonas* spp. in Fig. 8F and an increase in similarity in Fig. 8B.

Discussion

The quantitative data obtained in this investigation demonstrate the successful reduction of the bacterial load during the stages of evisceration. The data was utilized to build a biological map of the process and the additional microbiome profiles provided further insight into the organisms that were most prevalent through the evisceration process. In previous research, Zhang and others (2011) reported post chill results with an APC mean log 1.79 CFU/mL. Additionally, APC and *Enterobacteriaceae* exhibited post chill results of mean log 2.86 and 0.66 CFU/mL, respectively (Handley *et. al.*, 2010). An investigation on the effectiveness of chlorinated chill water, James and others (1992) reported post chill carcasses yielding mean log 2.51 CFU/mL for APC and mean log 1.75 CFU/mL for *Enterobacteriaceae*. Similarly, Berrang and Dickens (2000) noted APC pre-chill and post chill carcass counts of mean log 3.6 CFU/mL

and 2.9 CFU/mL. As for pre-chill, Bauermeister (2008) recovered APC mean log CFU/mL 4.24 from commercially processed carcasses.

Interestingly, the microbial counts obtained over these last 20 years have decreased as expected per changes in processing intervention strategies. Alternative antimicrobial strategies have been extensively investigated and are currently approved for the USDA Safe and Suitable List, such as formic acid, citric acid, lactic acid, propionic acid, peroxyacetic acid, tri-sodium phosphate, chlorine dioxide, acidified sodium chlorite and cetylpyridium chloride (Kim *et. al.*, 2017; Sofos *et. al.*, 2013; Bauermeister *et. al.*, 2008; Ricke *et. al.*, 2005; Ricke, 2003; Mulder et al., 1987; Izat et al., 1990; Dickens et al., 1994; Yang et al., 1998; Lillard, 1994; Doyle and Waldroup, 1996). These research studies and reviews have provided evidence that each antimicrobial has an optimal mode of application, such as dips, rinses, sprays or chill tank use. Additionally, each intervention can be more effective on certain bacterial groups than others. For instance, it has been noted previously that citric acid was more effective against Gram positive bacteria than Gram negative bacteria (Alonso-Hernando *et.al.*, 2009; Del Rio *et. al.*, 2007a, b). Hunter *et. al.* (2009) reported a reduction in the *Campylobacter* subspecies diversity from rehang to post chill using NGS.

More recently, microbiome analyses have been performed on the following poultry matrices, fecal, litter, carcasses, carcass weeps, and chlorinated chill tank water (Kim *et. al.*, 2017; Rothrock *et. al.*, 2016; Oakley *et al.*, 2013). These studies noted shifts in the microbiota through the production process and noted within this study. The multiple interventions in the slaughter and evisceration process reduced both the microbial load and the diversity of the microbiome. Kim *et. al.* (2017) observed a similar sample profile as this study, where 98.7% of the phyla present were identified as Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria,

and Cyanobacteria. The organisms present in this study have also been previously reported by other researchers analyzing meat sample microbiomes or from meat spoilage investigations (Kim *et. al.*, 2017; Rothcock *et. al.*, 2016; Handley *et. al.,* 2010; Nychas *et. al.*, 2008; Patsias *et. al.*, 2006; Borch *et. al.*, 1996). The presence of *Pseudomonas* in fresh carcasses is consistent with observations made by Hanning et al. (2009) when they used PCR to detect and differentiate *Pseudomonas* spp. from retail poultry carcasses. Additionally, *Pseudomonas* spp. have been found to differ between fresh versus refrigerated poultry meat (Arnaut-Rollier et al., 1999; Morales et al., 2016). In characterizing *Pseudomonas* recovered from spoiled poultry fillets, Morales et al (2016) observed considerable genotypic and phenotypic variability between and within species. Given the predominance of *Pseudomonas* observed in the current study and the genetic variability reported by Morales et al., (2016), whole genome sequencing of *Pseudomonas* spp. throughout processing and cold storage may reveal a pattern of particular strain succession during processing and cold storage. Likewise, the appearance of a particular strain at certain phases of processing may be indicative of the types of antimicrobials being employed. Finally, particular strains could be predictive indicators for increased likelihood of biofilm formation and/or favoring survival of certain foodborne pathogens such as *Campylobacter* (Hanning *et. al*., 2009; Hilbert *et. al.,* 2010; Morales *et. al*., 2016)

In conclusion, the evisceration process largely impacted the microbial diversity on carcass quality. This study identified the potential use of NGS in association with quantitative microbial data to determine the efficacy of a commercial antimicrobial multi-hurdle system. Additionally, broiler carcasses were characterized to establish a typical commercial microbiome profile. As for the identification of potential indicator organisms, *Pseudomonas,*

Enterobacteriaceae, and *Weeksellaceae Chryseobacterium* were identified as potential indicator

organisms because they were isolated from all processing abattoirs and sampling locations.

Acknowledgements

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Figure Legends

Figure 1. Diagram of the broiler slaughter process. The stars represent sampling locations.

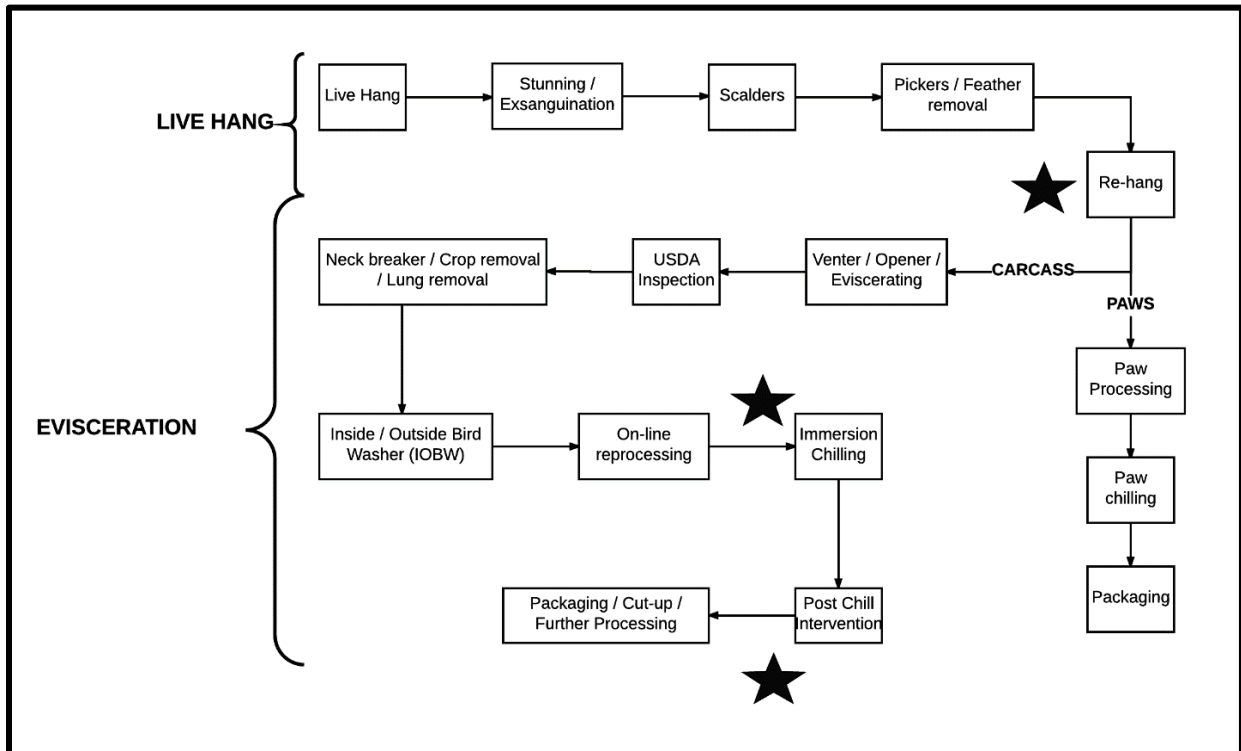**Figure 2**. Bio-map of evisceration. The bacterial mean log CFU/mL counts for both *Enterobacteriaceae* and aerobic plate counts (APC)

Figure 3. All phylum present through re-hang, pre-chill, and post Chill.

Figure 4. Bio-map of microorganisms through evisceration when the genera were identified at all sampling locations and abattoirs.

Figure 5. Chao 1 rarefaction curve. The measure of richness within a community at each processing abattoir and testing location within the plant.

Figure 6. OTU rarefaction curves. The number of observed OTU's versus the length of sequence read at each processing plant and testing location within the plant.

Figure 7. Beta diversity between sampling locations and individual processing abattoir. Weighted and unweighted UniFrac PCoA plots A) Plant 1 weighted. B) Plant 1 unweighted. C) Plant 2 weighted. D) Plant 2 unweighted. E) Plant 3 weighted. F) Plant 3 unweighted. Orange is for rehang, blue is for pre-chill, and red is for post chill.

Figure 8. Beta diversity among *Pseudomonas* spp. between sampling locations and individual processing abattoir. Weighted and unweighted UniFrac PCoA plots A) Plant 1 weighted. B) Plant 1 unweighted. C) Plant 2 weighted. D) Plant 2 unweighted. E) Plant 3 weighted. F) Plant 3 unweighted. Orange is for rehang, blue is for pre-chill, and red is for post chill.

Table 1. Microbial log CFU/mL reduction on Whole Bird Carcasses Rinses

Step	Plant A		Plant B		Plant C		All Plant	
	APC Mean	EB Mean	APC Mean	EB Mean	APC Mean	EB Mean	APC Mean	EB Mean
Rehang	4.92 ± 0.28 a*	3.37 ± 0.24 a	4.52 ± 0.20 a	2.64 ± 0.28 a	4.45 ± 0.29 a	2.94 ± 0.20 a	4.63 ± 0.33 a	2.99 ± 0.38 a
Pre-chill	3.94 ± 0.50 b	2.65 ± 0.44 b	2.83 ± 0.62 b	1.14 ± 0.94 b	2.69 ± 0.12 b	1.59 ± 0.26 b	3.15 ± 0.73 b	1.79 ± 0.88 b
Post Chill	1.12 ± 0.96 c	0.19 ± 0.42 c	0.90 ± 0.51 c	0.16 ± 0.29 c	0.40 ± 0.36 c	0.00 ± 0.00 c	0.81 ± 0.71 c	0.12 ± 0.30 c

* For each individual plant, testing locations that do not share a similar letter designation are significantly different with a p-Value <0.01.

Table 2. Tukey-Kramer HSD for all plant microbial counts log CFU/mL.

Tukey-Kramer HSD		APC		Enterobacteriaceae	
Level	- Level	Difference	*p*-Value	Difference	*p*-Value
Rehang	Post Chill	3.82	<0.001	2.86	<0.001
Pre-chill	Post Chill	2.34	<0.001	1.67	<0.001
Rehang	Pre-chill	1.47	<0.001	1.19	<0.001

Table 3. All identified microorganisms present at post chill and processing abattoirs

OTU ID	All Plant Mean % Abundance
Pseudomonas	83.51
Enterobacteriaceae	2.23
Bacteroides	1.46
Chryseobacterium	1.13
Flavobacterium	0.37
Moraxellaceae	0.36
Aeromonadaceae	0.30
Ruminococcaceae	0.21
Clostridium	0.20
Mycoplana	0.14
Psychrobacter	0.14
Oxalobacteraceae	0.13
Acinetobacter	0.12
Sphingobacterium	0.10
Microvirgula	0.06
Pseudomonadaceae	0.06
Paenibacillus	0.04
Comamonadaceae	0.03
Lachnospiraceae	0.03
Clostridiaceae	0.02
Gammaproteobacteria Other	0.02
Clostridiaceae Other	0.01
Pelosinus	0.01

Table 4. List of microorganisms present during all testing locations and abattoirs are in bold. Species identified when the genera were present in >1.0% relative abundance at post chill.

#OTU ID	Rehang % Abundance	Pre-chill % Abundance	Post chill % Abundance
Enterobacteriaceae	4.3	5.8	2.2
Citrobacter	0.0	0.0	0.0
Erwinia	0.6	0.0	
Enterobacteriaceae Other	0.1	0.1	0.0
Serratia	0.0	0.0	
Yersinia	0.0	0.0	
Pseudomonadaceae	1.7	0.1	0.1
Pseudomonadaceae Other	0.0	0.0	0.0
Pseudomonas	45.9	60.6	71.7
Pseudomonas fragi	0.0		0.0
Pseudomonas Other	10.7	1.9	23.0
Pseudomonas veronii	0.5	0.2	0.3
Pseudomonas viridiflava	1.3	0.3	0.1
Chryseobacterium	4.0	0.2	1.1
Sphingobacterium faecium	7.5	0.9	0.1
Aeromonadaceae	6.8	4.5	0.3
Microvirgula	4.3	9.1	0.1

Table 5. Summary of Illumina Mi-Seq Read Lengths and QIIME Shannon Diversity

Alpha Diversity			
Sample Location	Read Length	Shannon	Standard Deviation
Plant 1			
Re-hang	38000	2.98	0.51
Pre-Chill	38000	2.94	0.42
Post Chill	38000	0.85	0.64
Plant 2			
Re-hang	40000	2.52	0.54
Pre-Chill	40000	2.05	1.56
Post Chill	40000	1.50	2.19
Plant 3			
Re-hang	62000	1.90	0.30
Pre-Chill	62000	1.27	0.54
Post Chill	62000	NA	NA

Figure 1. Diagram of the broiler slaughter process. The stars represent sampling locations.

Figure 2. Evisceration microbial reduction. The bacterial mean log CFU/mL counts for both *Enterobacteriaceae* and aerobic plate counts (APC)
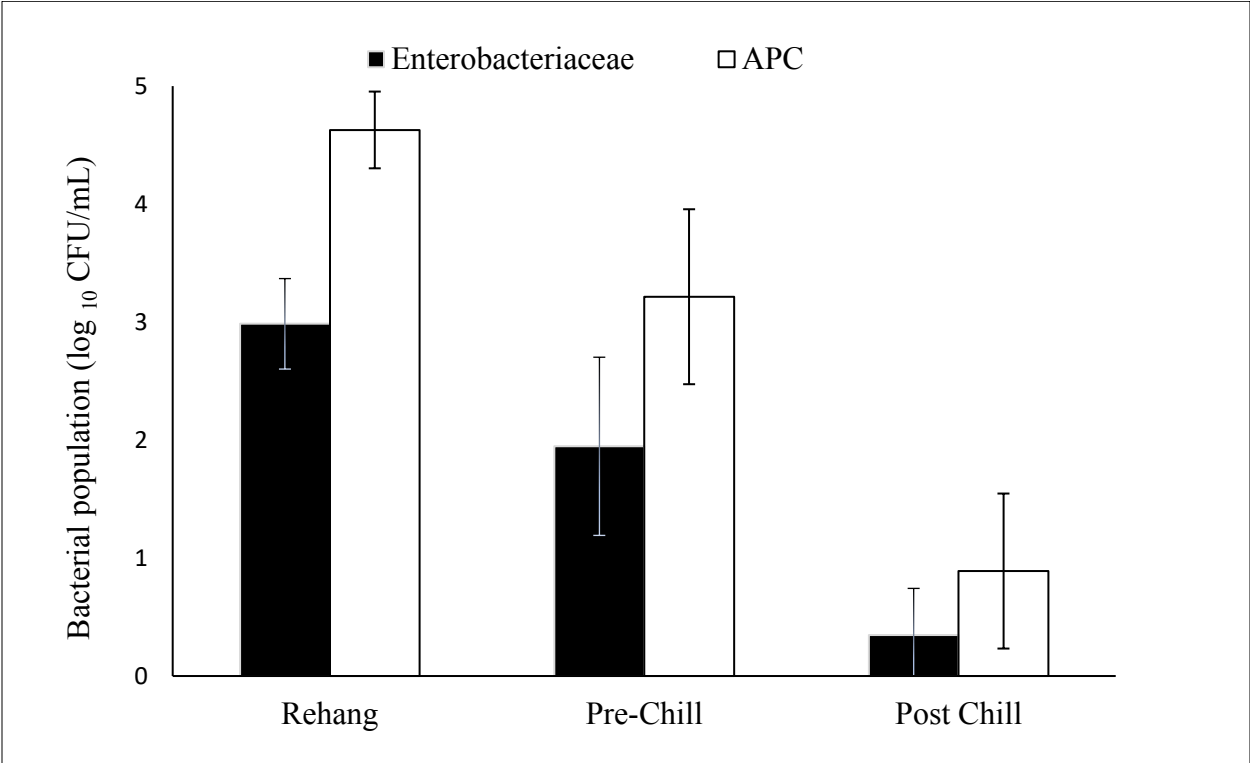
Figure 3. All phylum present through re-hang, pre-chill, and post chill.
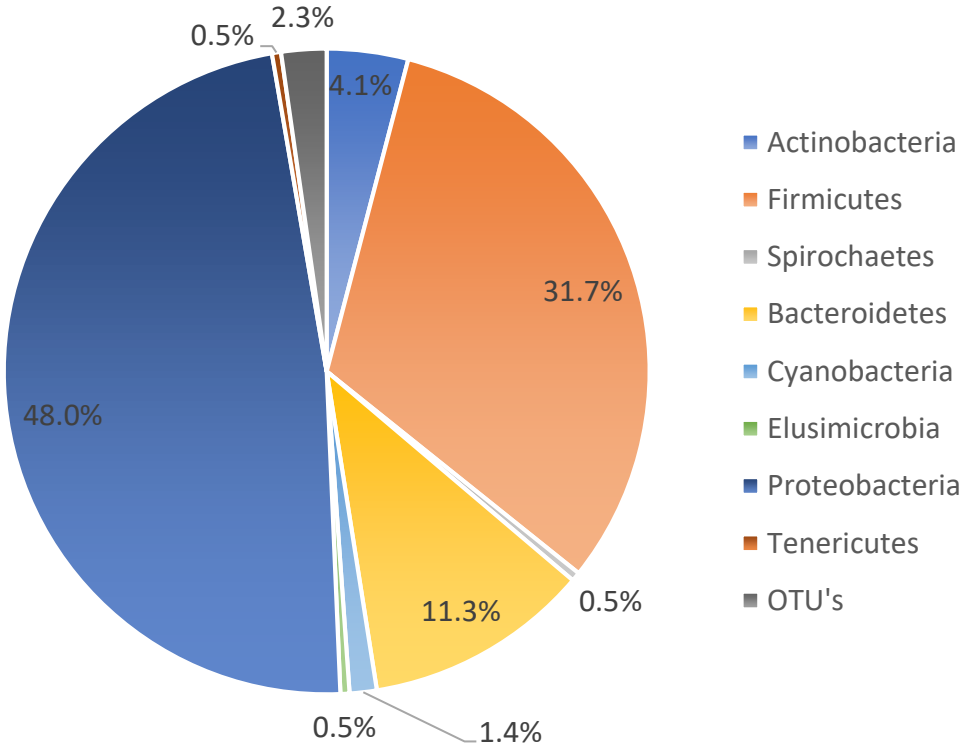
Figure 4. Genera present during all sampling stages at all processing abattoirs.
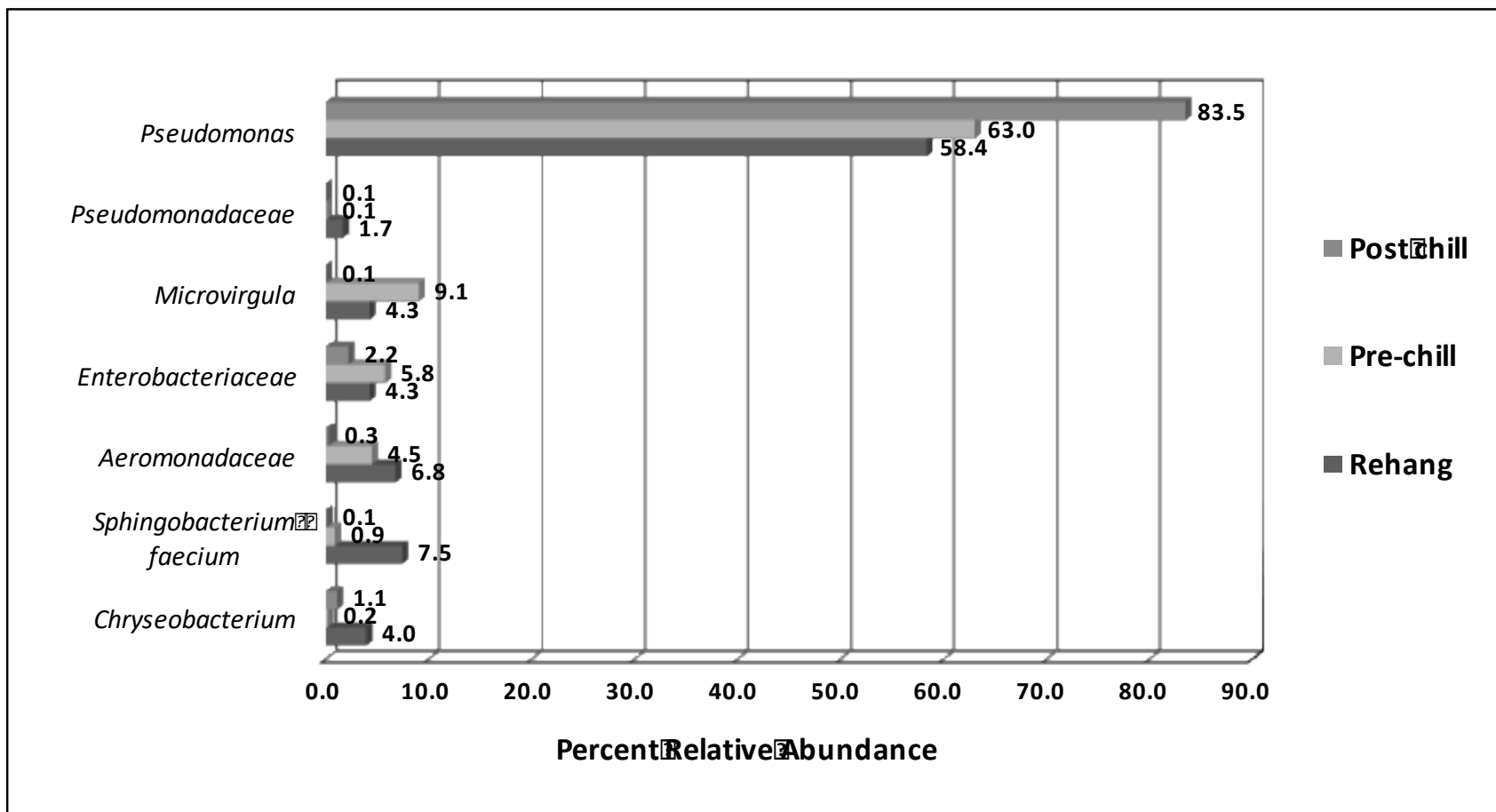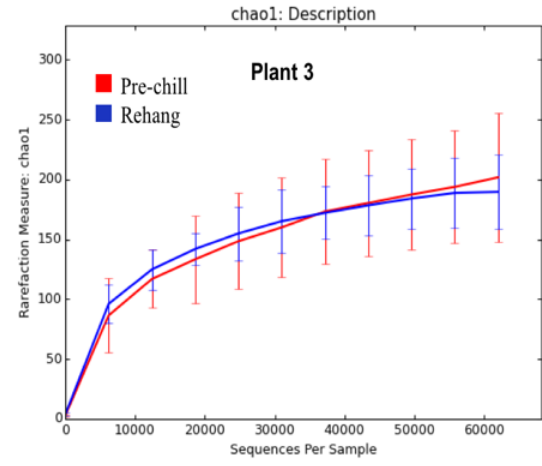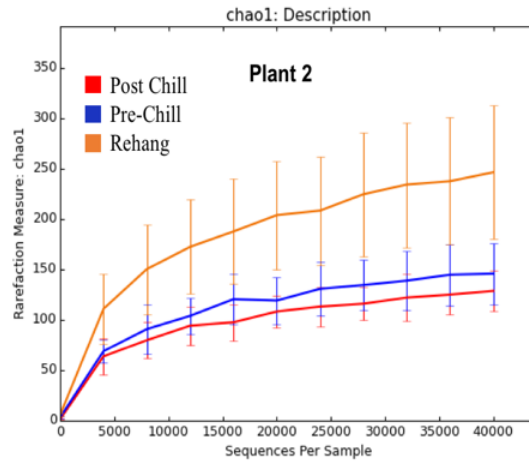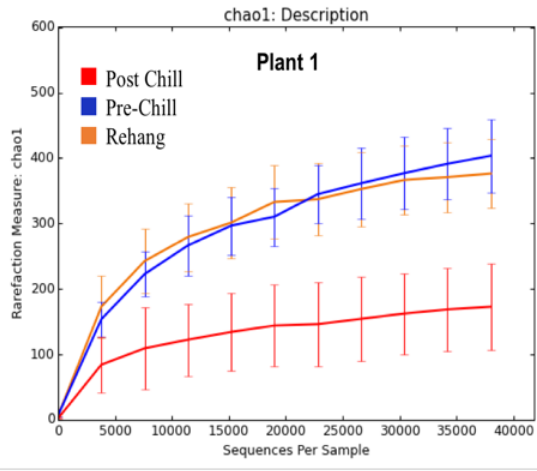
Figure 5. Chao 1 rarefaction curve.

Figure 6. OTU rarefaction curves.
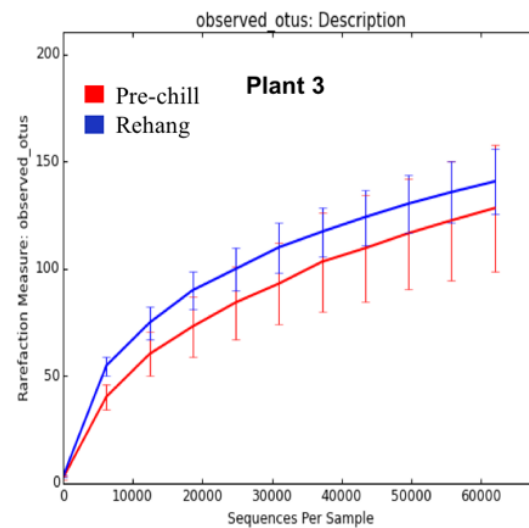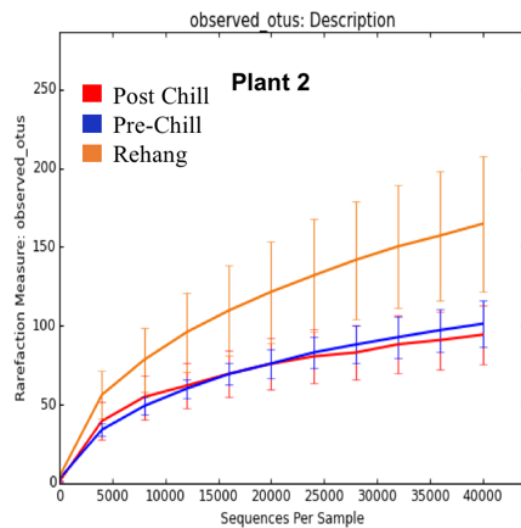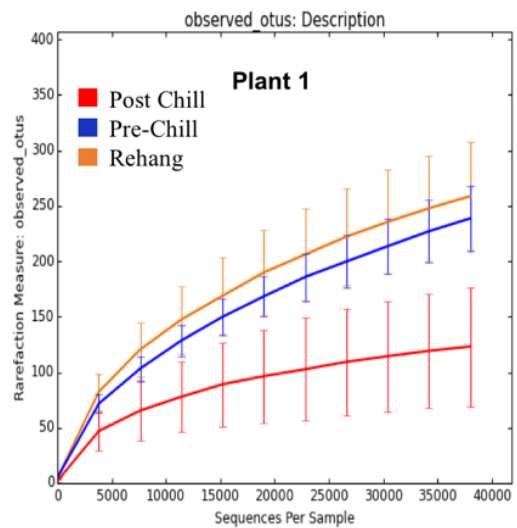
Figure 7. Beta diversity between sampling locations and individual processing abattoir. Weighted and unweighted UniFrac PCoA plots A) Plant 1 weighted. B) Plant 1 unweighted. C) Plant 2 weighted. D) Plant 2 unweighted. E) Plant 3 weighted. F) Plant 3 unweighted. Orange is for rehang, blue is for pre-chill, and red is for post chill.
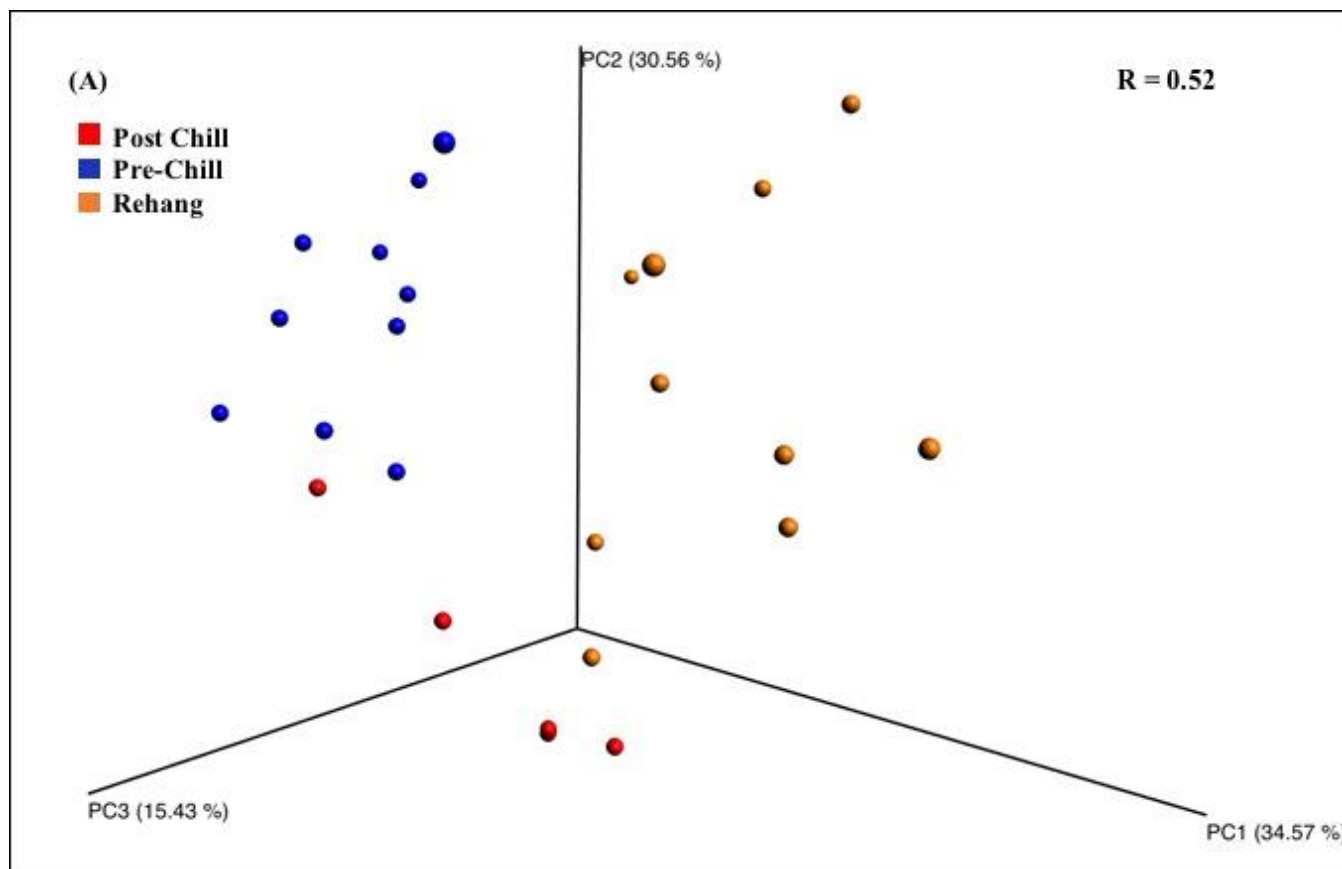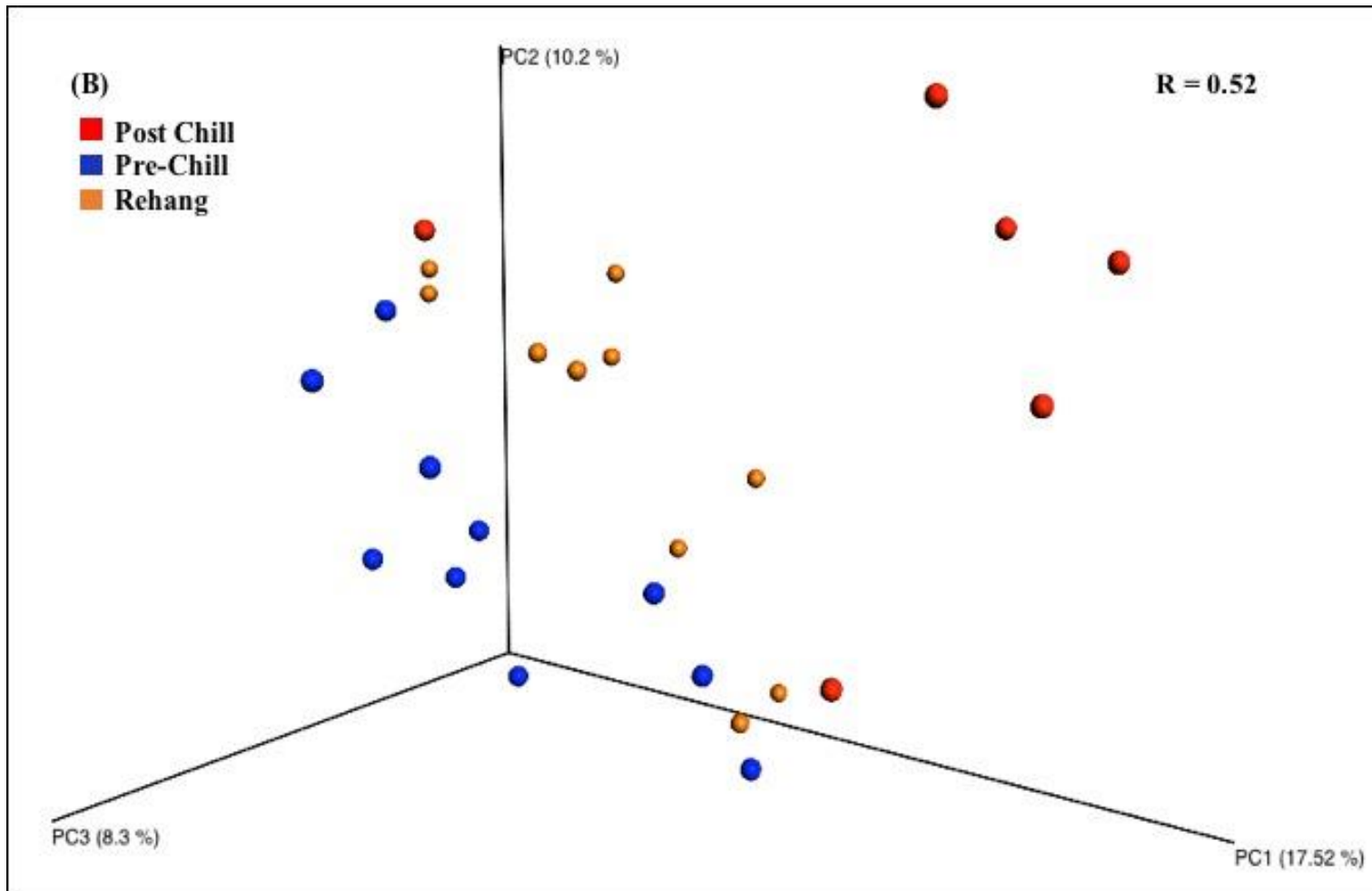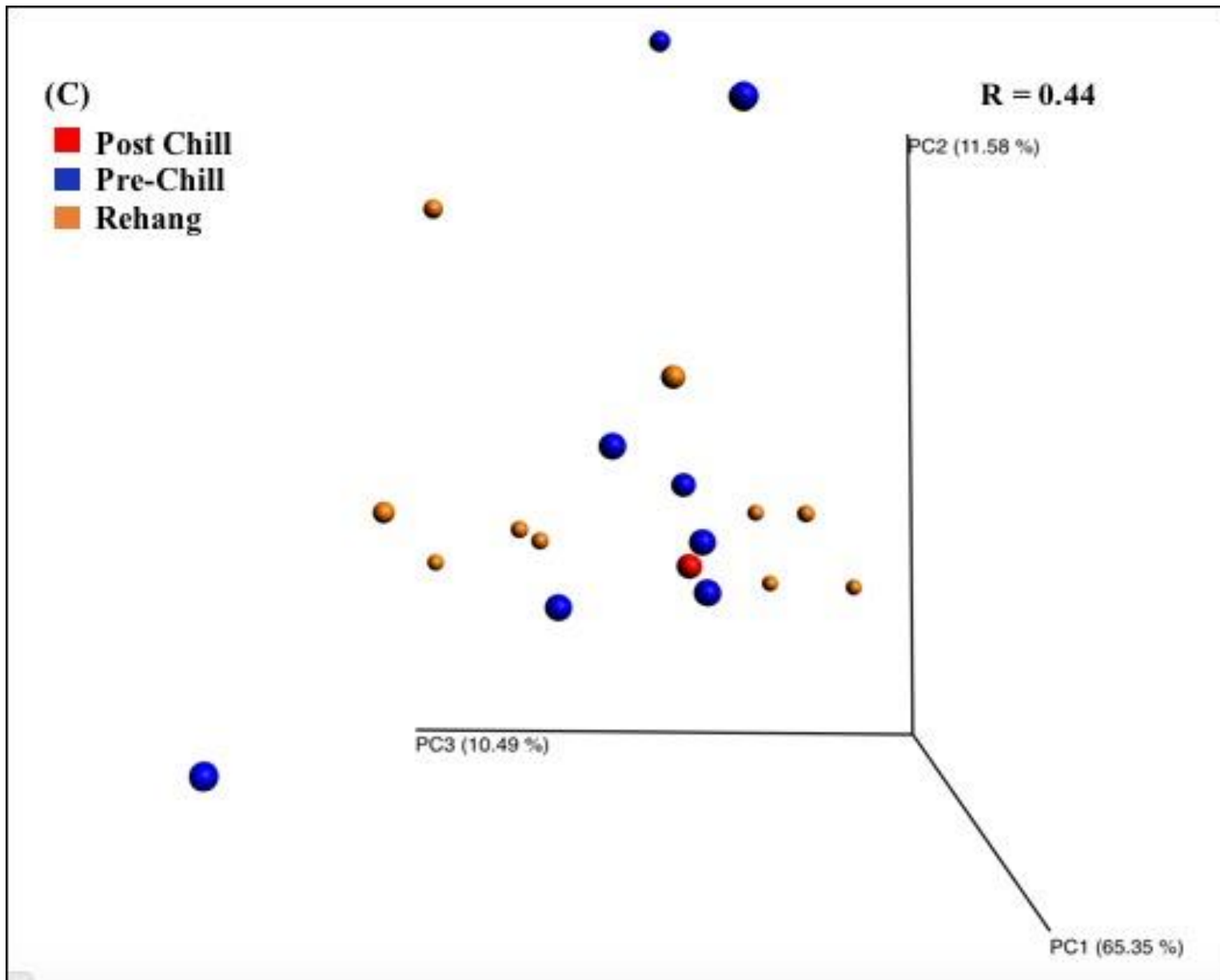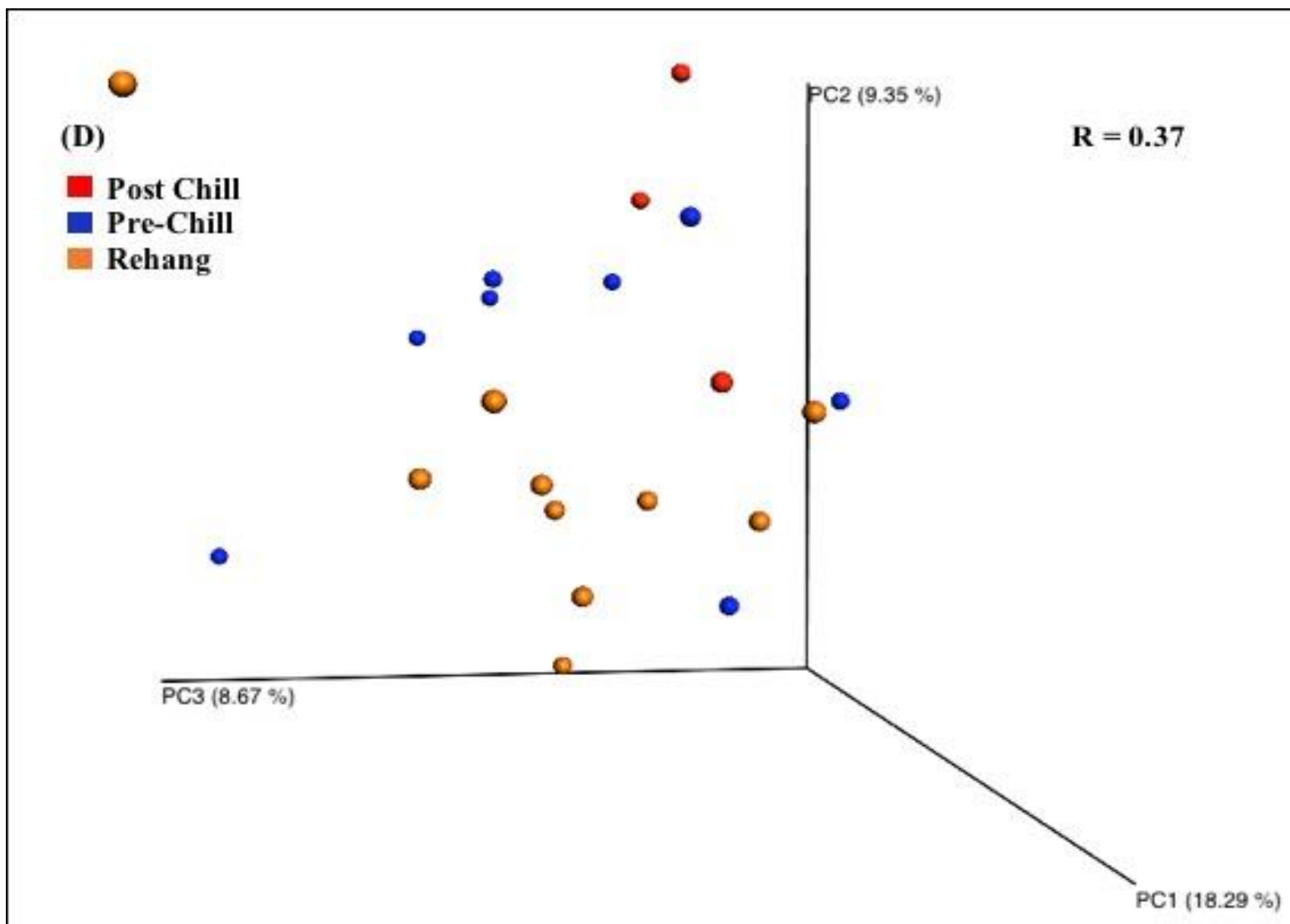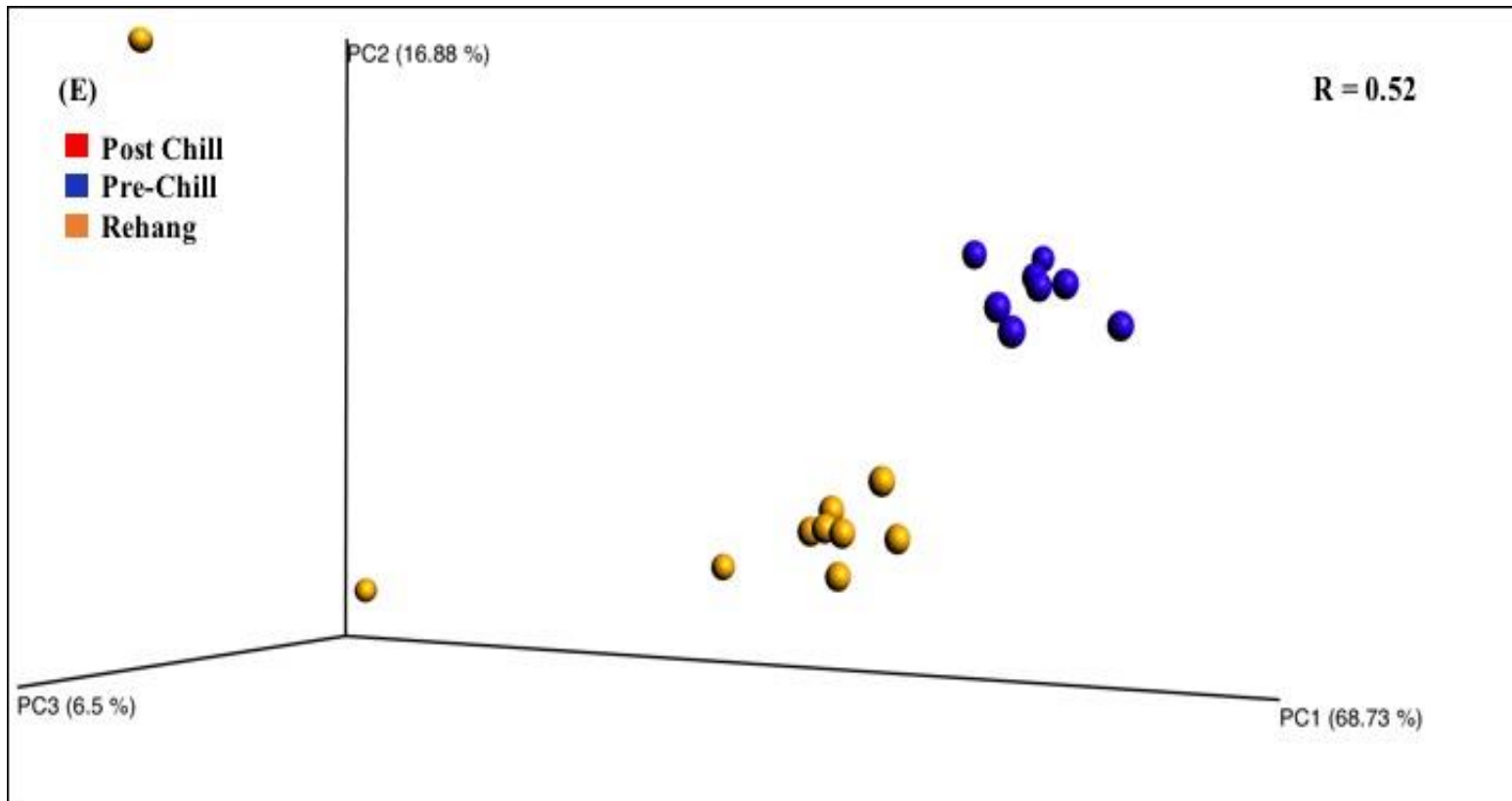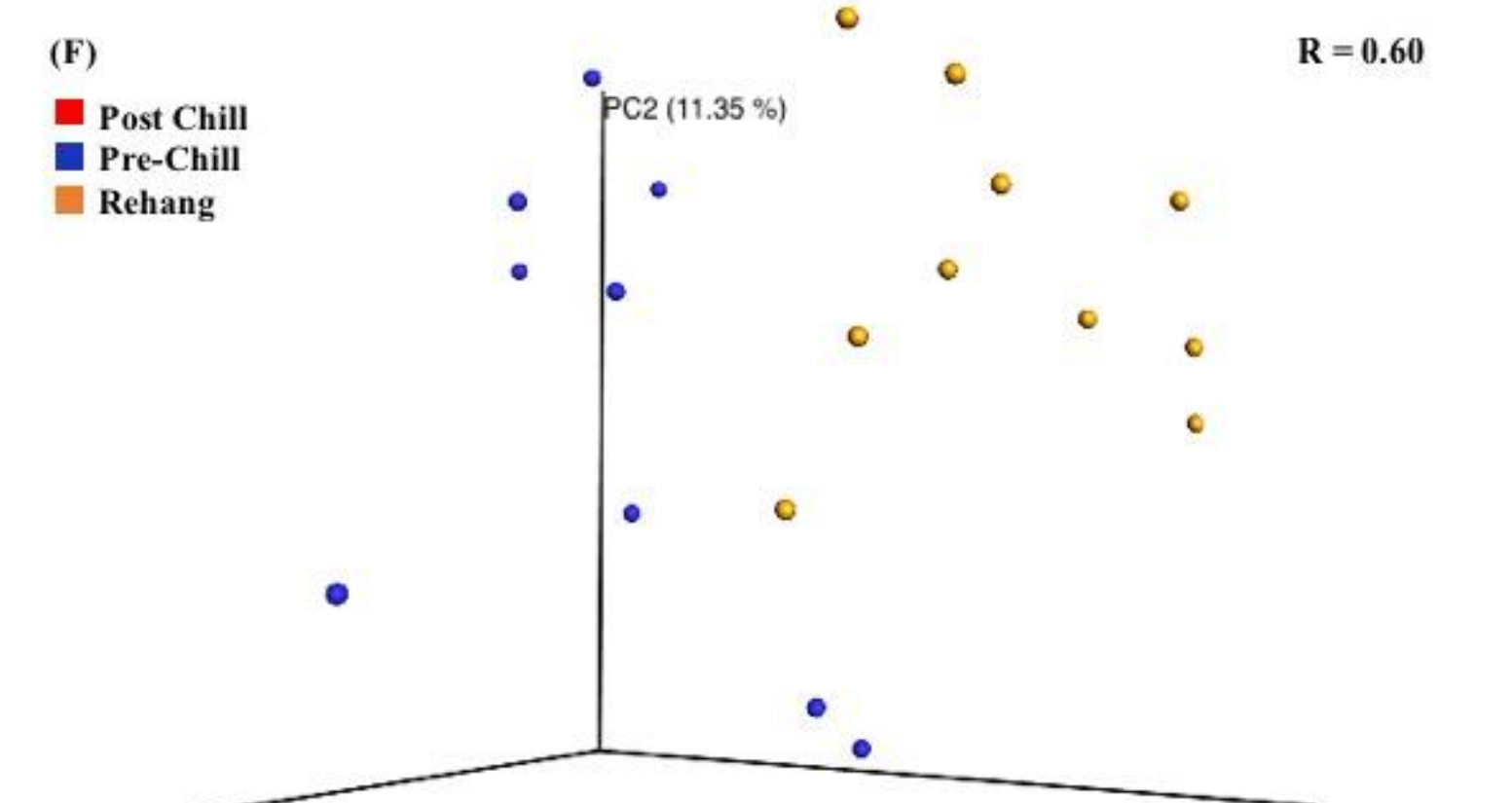
Figure 8. Beta diversity among *Pseudomonas* sp. between sampling locations and individual processing abattoir. Weighted and unweighted UniFrac PCoA plots A) Plant 1 weighted. B) Plant 1 unweighted. C) Plant 2 weighted. D) Plant 2 unweighted. E) Plant 3 weighted. F) Plant 3 unweighted. Orange is for rehang, blue is for pre-chill, and red is for post chill.
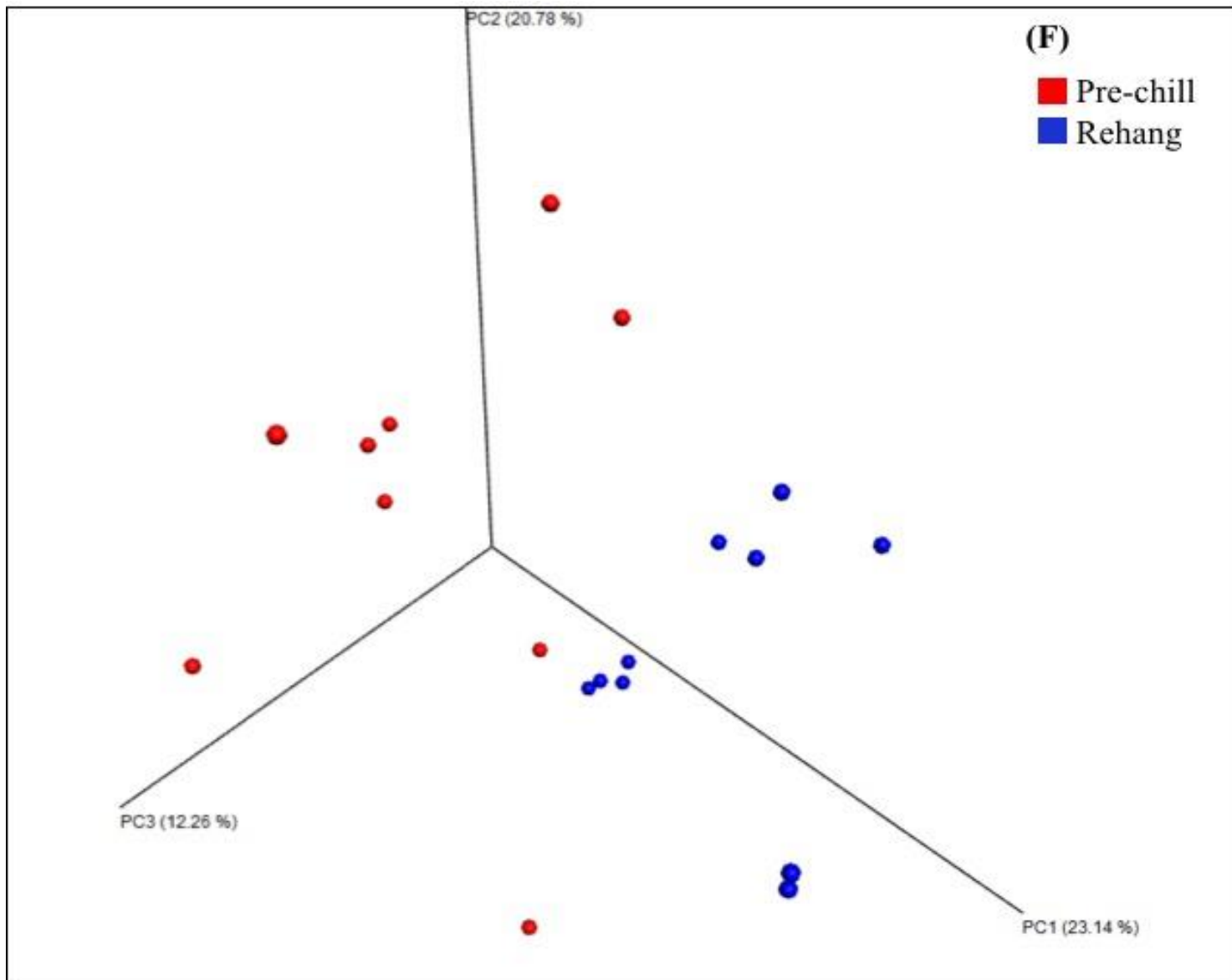
PC2 (20.78 %)

(F)

■ Pre-chill
■ Rehang

PC3 (12.26 %)

PC1 (23.14 %)

130
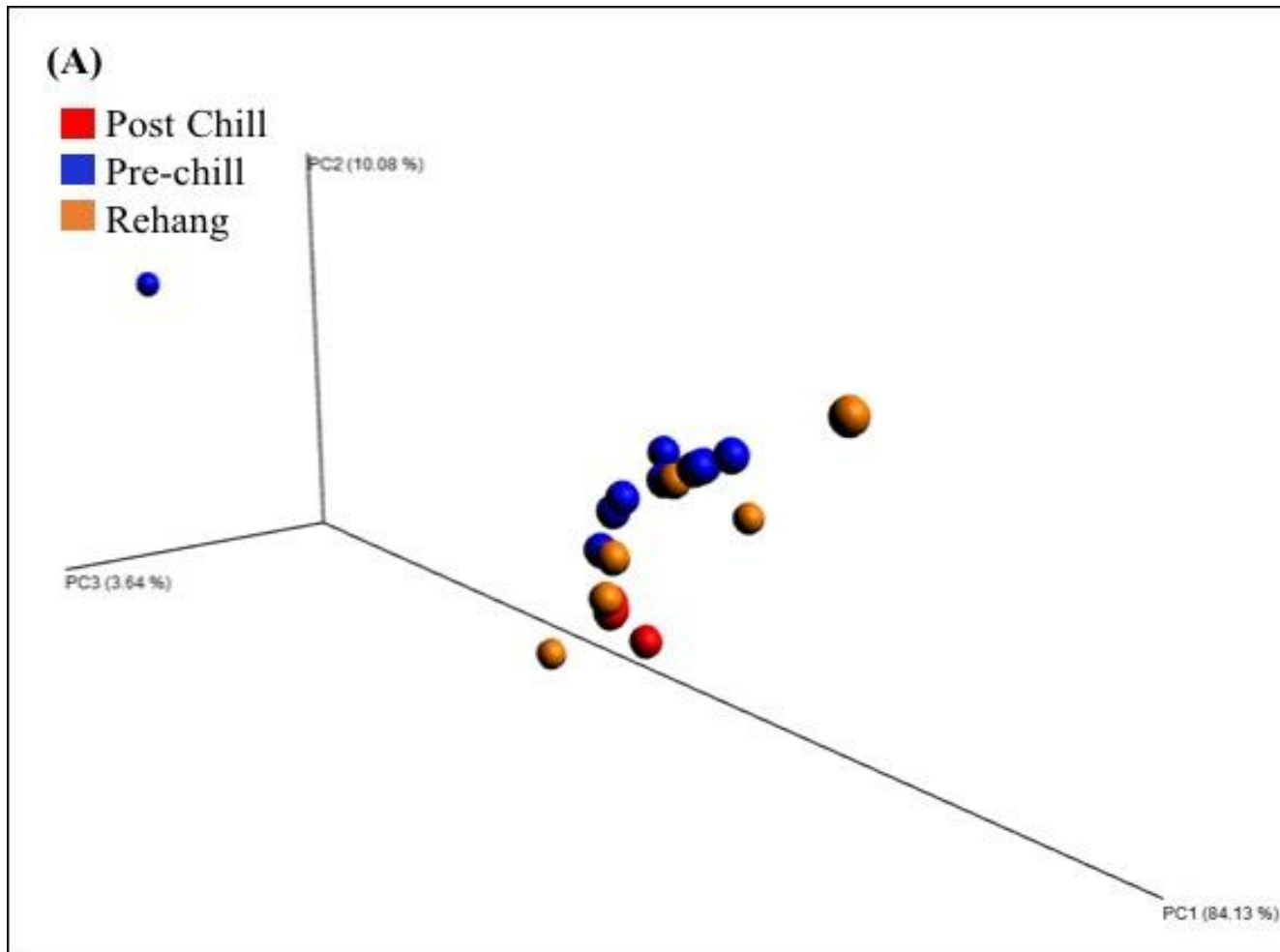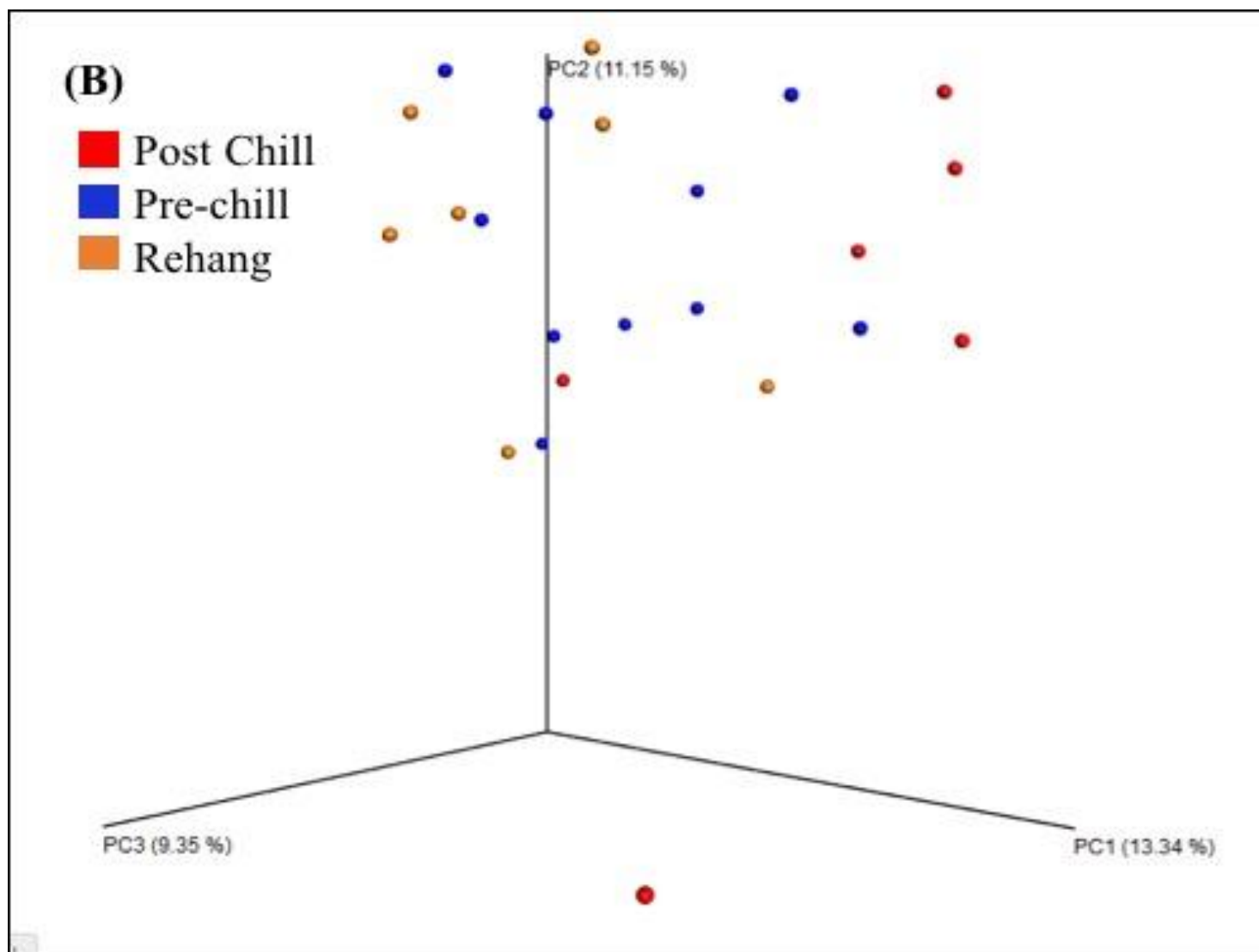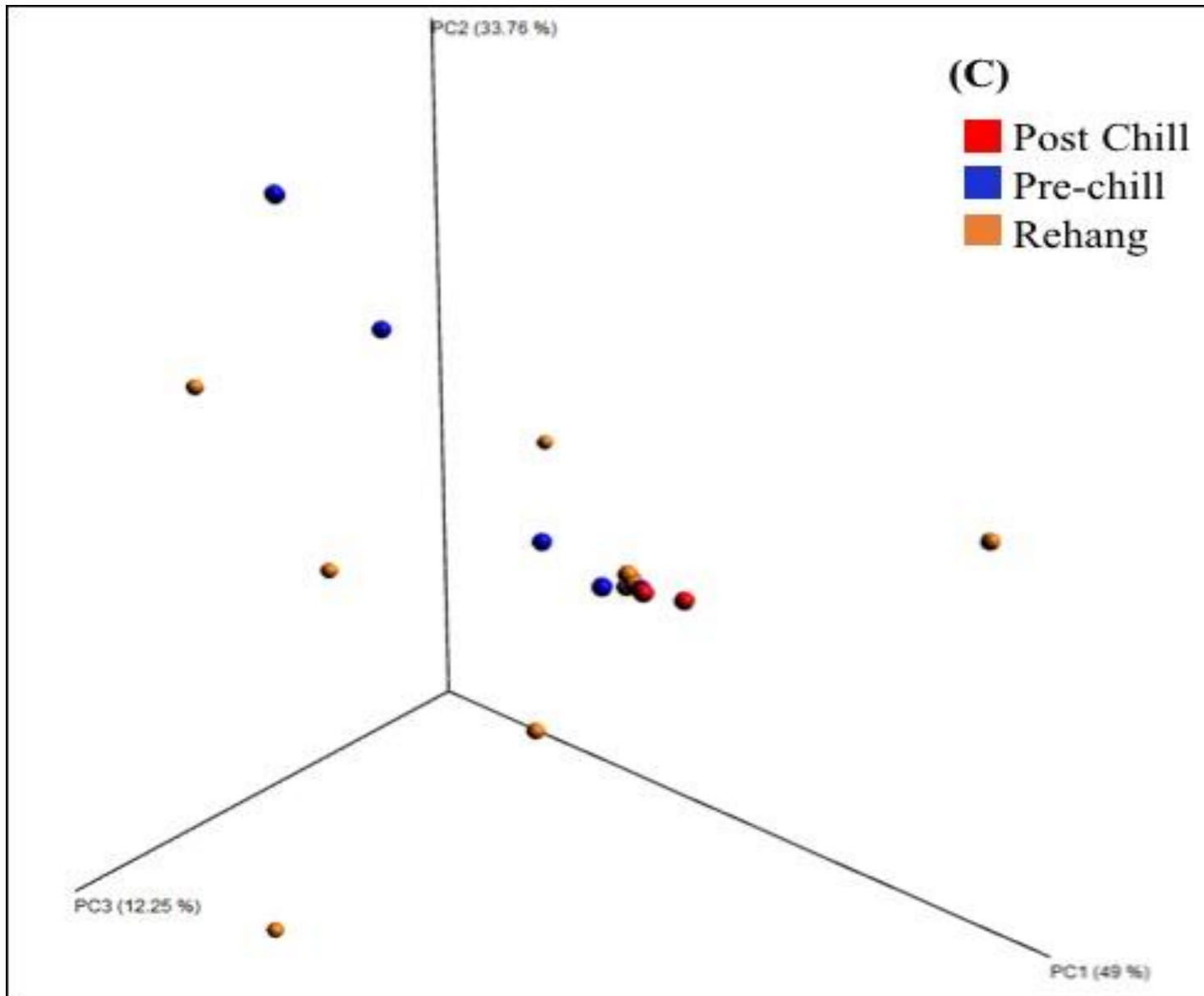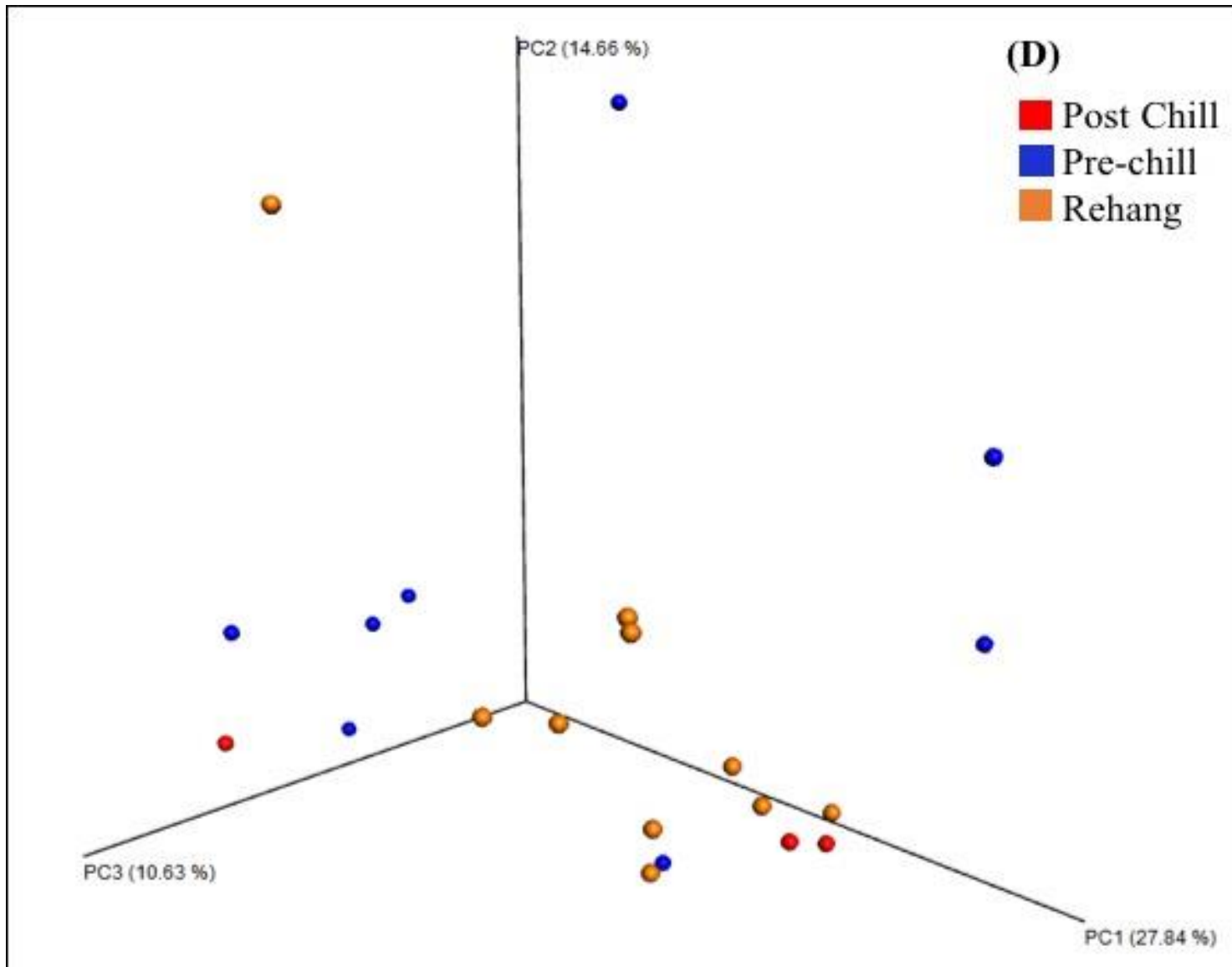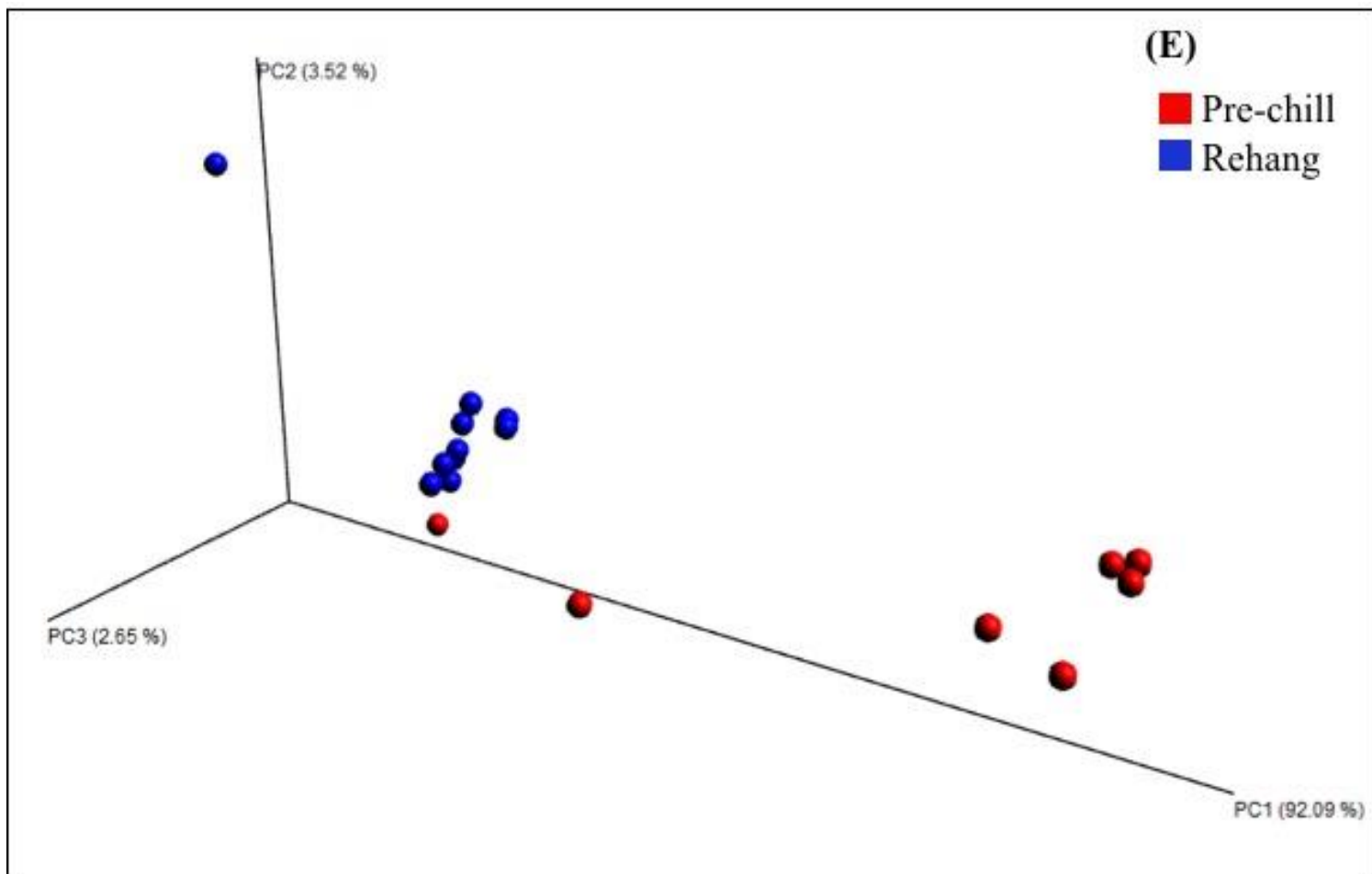
Chapter 4

Conclusion

As broilers traverse the farm to fork continuum, the complex variables associated with live production and evisceration impact the microbiota of raw post chill chicken carcasses. These microorganisms may become etiological agents associated with food borne illness or meat spoilage. There are strong indications that horizontal transmission contributes to the distribution of bacteria throughout the various phases in the production system. In order for researchers and industry processors to continue developing and implementing new antimicrobial reduction strategies, establishing an indicator organism could prove to be beneficial. Such an organism would behave similarly to pathogens of interest when exposed to environmental stimuli. Additionally, the indicator organism could be utilized in studies performed in commercial processing facilities.

High throughput sequencing of 16S rRNA genes has become more available to the research community and have begun demonstrating the impact characterizing the microbiota of several sample types through farm to fork continuum. NGS is the future in microbiome analysis and provides more detailed data on the effects antimicrobial hurdle may have on one or more taxonic groups.

The current study identified the potential use of NGS in association with quantitative microbial data to determine the efficacy of poultry processing systems. In this study, commercially processed carcass microbiomes were characterized to establish a typical processing microbiome profile. Quantitative data indicated that the production systems reduced the microbial load as expected. The microbiota also reflected a similar reduction where the WBCR identified 222 Operational Taxonomic Units' (OTU's) at rehang and of which only 23 OTU's or 10% of the population was recovered post chill. Microbiome data suggested a high

relative abundance of *Pseudomonas* at post chill and indicated other potential indicator

organisms to be Enterobacteriaceae, *Bacteroidetes*, Weeksellaceae, and *Chryseobacterium* due to

having been isolated from all processing abattoirs and sampling locations.
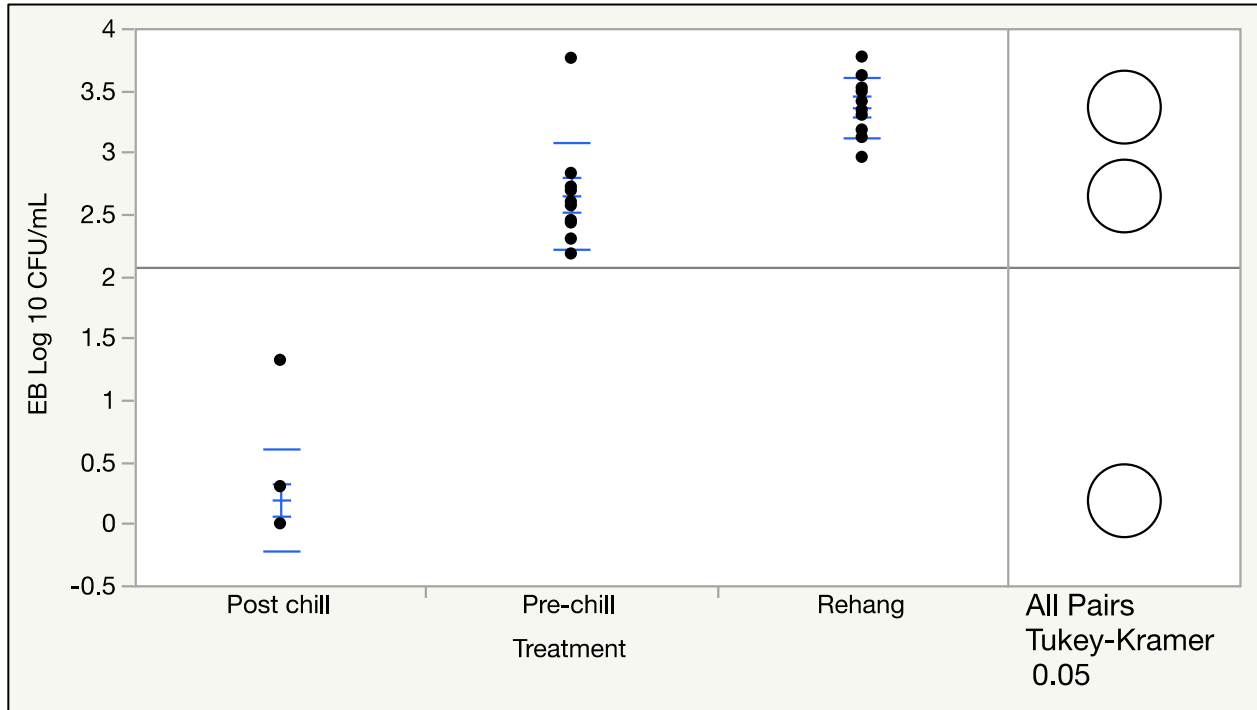
DISSERTATION APPENDICES

Figure 1. Plant A rehang, pre-chill, and post chill APC Tukey HSD report with means and standard deviation table from JMP.
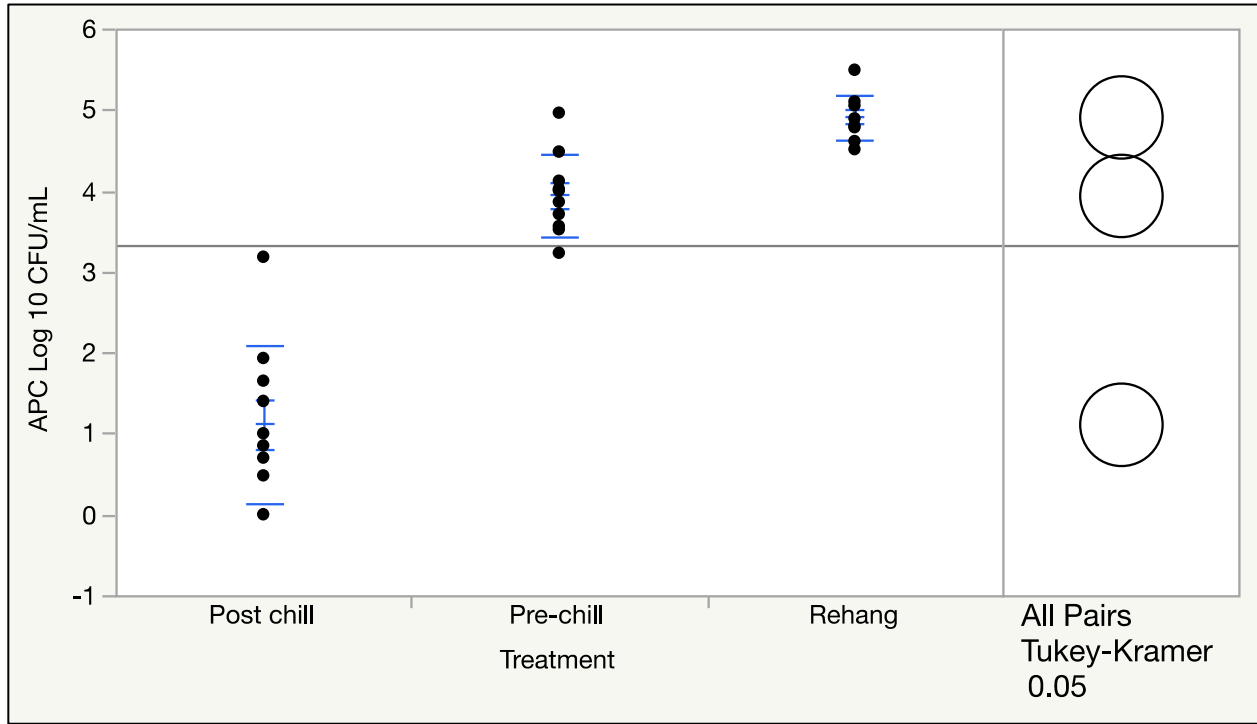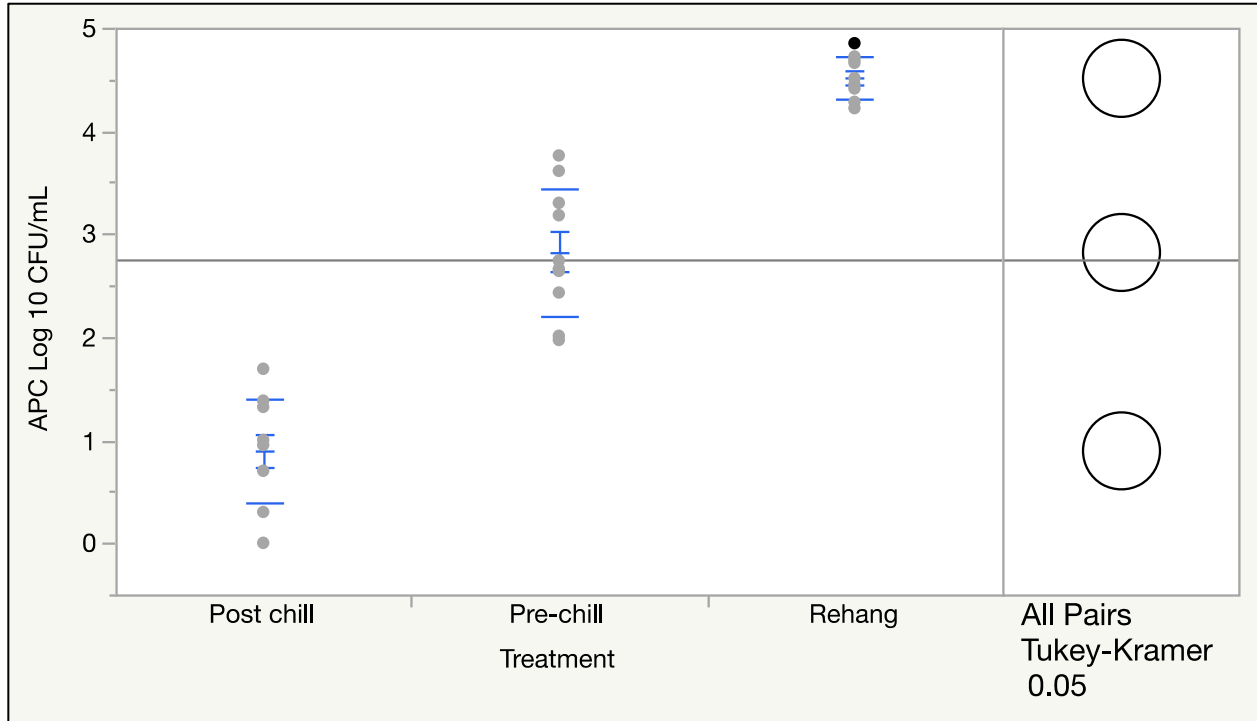

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	3.797000	0.2904373	3.076885	4.517115	<.0001*
Pre-chill	Post chill	2.827000	0.2904373	2.106885	3.547115	<.0001*
Rehang	Pre-chill	0.970000	0.2904373	0.249885	1.690115	0.0067*

Figure 2. Plant A rehang, pre-chill, and post chill Enterobacteriaceae Tukey HSD report with means and standard deviation table from JMP.
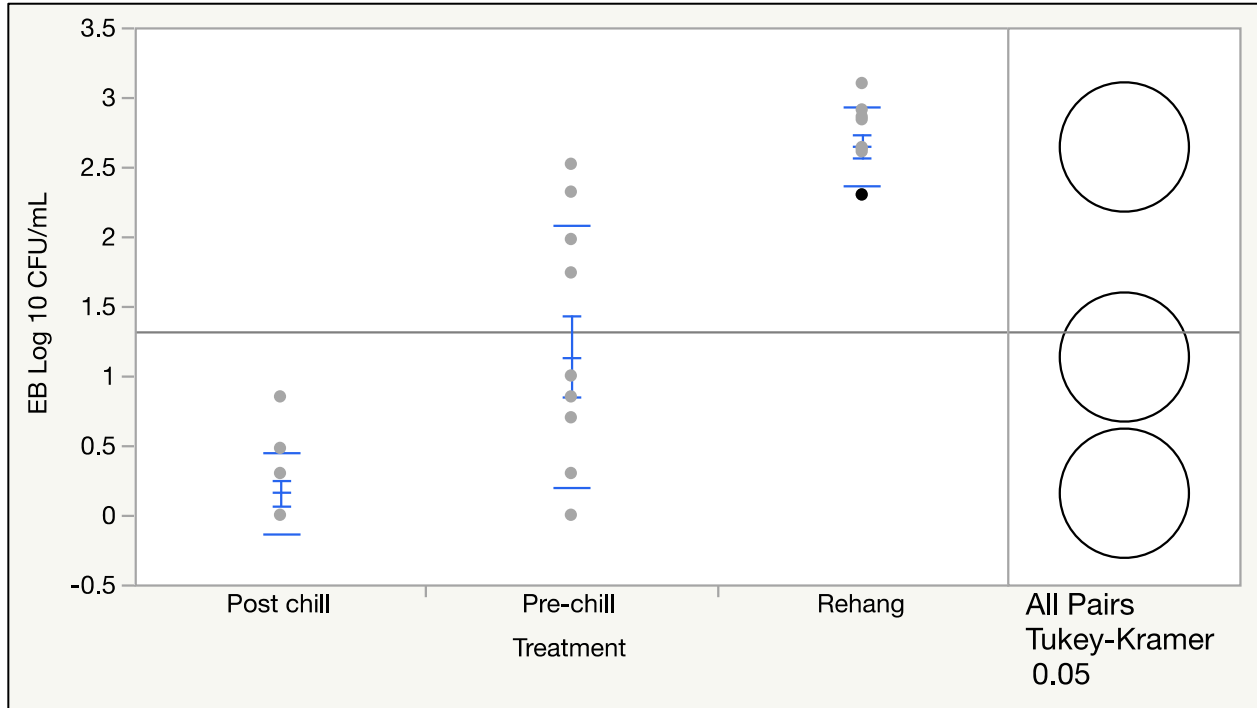

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	3.179000	0.1677489	2.763080	3.594920	<.0001*
Pre-chill	Post chill	2.461000	0.1677489	2.045080	2.876920	<.0001*
Rehang	Pre-chill	0.718000	0.1677489	0.302080	1.133920	0.0006*

Figure 3. Plant B rehang, pre-chill, and post chill APC Tukey HSD report with means and standard deviation table from JMP.
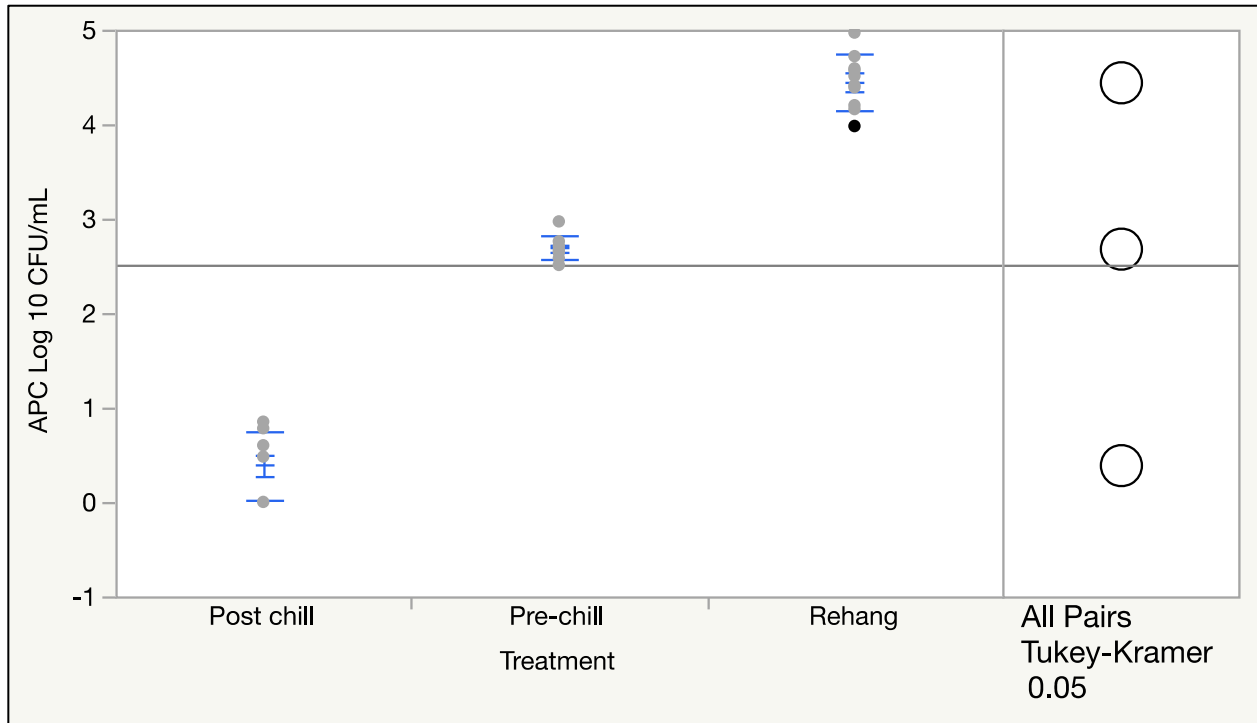

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	3.616000	0.2132507	3.087262	4.144738	<.0001*
Pre-chill	Post chill	1.926000	0.2132507	1.397262	2.454738	<.0001*
Rehang	Pre-chill	1.690000	0.2132507	1.161262	2.218738	<.0001*

Figure 4. Plant B rehang, pre-chill, and post chill Enterobacteriaceae Tukey HSD report with means and standard deviation table from JMP.
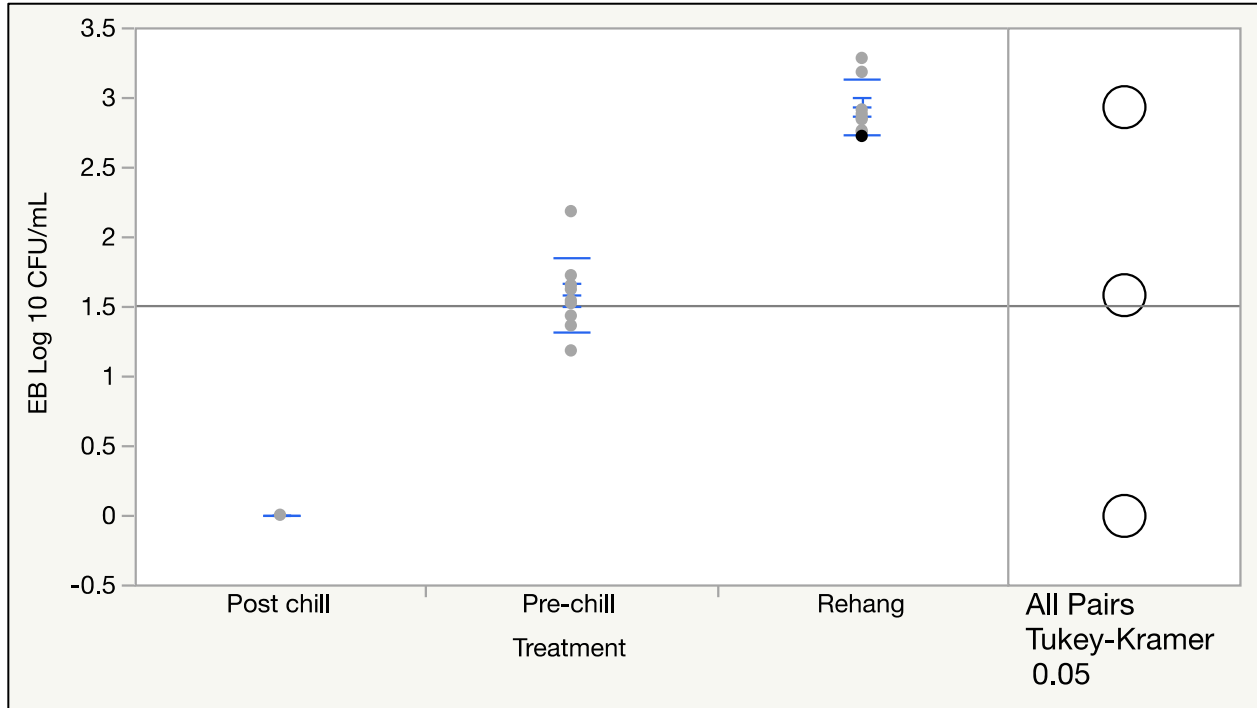

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	2.486000	0.2647373	1.829606	3.142394	<.0001*
Rehang	Pre-chill	1.508000	0.2647373	0.851606	2.164394	<.0001*
Pre-chill	Post chill	0.978000	0.2647373	0.321606	1.634394	0.0028*

Figure 5. Plant C rehang, pre-chill, and post chill APC Tukey HSD report with means and standard deviation table from JMP.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	4.053000	0.1240161	3.745512	4.360488	<.0001*
Pre-chill	Post chill	2.292000	0.1240161	1.984512	2.599488	<.0001*
Rehang	Pre-chill	1.761000	0.1240161	1.453512	2.068488	<.0001*

Figure 6. Plant C rehang, pre-chill, and post chill Enterobacteriaceae Tukey HSD report with means and standard deviation table from JMP.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Rehang	Post chill	2.935000	0.0859328	2.721937	3.148063	<.0001*
Pre-chill	Post chill	1.585000	0.0859328	1.371937	1.798063	<.0001*
Rehang	Pre-chill	1.350000	0.0859328	1.136937	1.563063	<.0001*

Figure 7. Curriculum vitae

John A Handley III

██████████
████████████
█████████
████████

EDUCATION

August 2010 – Present, University of Arkansas, Fayetteville, AR
- Ph.D. Candidate for Cellular and Molecular Biology
- Dissertation: Microbiome of Commercial Broilers through Evisceration and Immersion Chilling
- Dissertation Advisors: Steven C. Ricke, Ph.D., Ravi D. Barabote, Ph.D., Bennie J. Bench, Ph.D., Young Min Kwon, Ph.D., Jeffrey A. Lewis, Ph.D.

June 2011, Advanced HACCP Administrator Course
- International HACCP Alliance

August 2006 – August 2010, University of Arkansas, Fayetteville, AR
- M.S. Food Science
- Thesis: Microbial Ecology of Whole Poultry Carcasses Stored at Room Temperature in Commercial Processing Combos
- Thesis Advisor: Steven C. Ricke, Ph.D., Robert O. Apple, Ph.D., Michael G. Johnson, Ph.D., Frank T. Jones, Ph.D.

August 2001 – December 2005, University of Arkansas, Fayetteville, AR
- B.S. Biology

EMPLOYMENT

Nov. 2016 – Current, Cedar Lake Products, Inc.
Director of Food Safety and Quality Assurance

Manage and provide direction on all matters pertaining to food safety and quality control. Perform annual audits to ensure that the manufacturing facilities are in compliance with customer requirements and regulatory agencies. Provide consulting services for processors regarding the implementation of GFSI food safety schemes, SSOP and GMP related activities that mitigate food safety risks associated with daily production activities. Develop, implement, and review data over SSOP and GMPs for production plants.

Feb. 2015 – Nov. 2016, Tyson Foods, Corporate Food Safety Research Laboratory
Food Safety and Technical Auditor

Perform detailed documented audits for plant laboratory procedures that verify the plants are compliant with government and/or FSQA Corporate policies and

calibrating lab equipment while on-site. Both assist and instruct a Microbiology Laboratory course to Tyson Foods personnel. Also, share responsibility in conducting proficiency testing program for Prepared Foods and Poultry Prepared business units. Developing and implementing emergency on-site and remote computer training of plant personnel. Other duties include teaching and customizing LabWare LIMS computer software, consulting with plant personnel in regards to government regulations or Corporate FSQA programs or policies, and lab procedures. Provide trouble shooting assistance to processing facilities, which includes writing detailed reports covering the finding and recommendations of the lab team. Maintaining public folders of the lab manual, plant audits, and proficiency testing files as well maintaining and optimizing LIMS database for all Tyson Foods production plants. Knowledge of ISO Quality System principles and tasks including training, calibration, internal ISO audits of corporate and regional labs.

Jan. 2014 – Feb. 2015, Tyson Foods, Raw and Further Processing Plant
Food Safety Quality Assurance Supervisor

Managed a team of FSQA technicians to ensure the plant had appropriate FSQA presence during operations. Responsible for interviewing, monitoring, and administering the job duties for FSQA hourly personnel. This includes monitoring and maintaining Quality Assurance programs to verify that product specifications are met, communicating with all levels of management and USDA, and ensuring compliance with applicable regulatory requirements. Performed GMP audits of various plant departments and worked with maintenance and production on a joint task force to reduce potential foreign material incidence. Assisted in multiple FSA, FSQA, and BRC audits by collecting and reviewing Plant.View data along with reviewing and implementing new food safety and quality plant programs to ensure alignment with corporate mandated policies. Reviewed specifications and production data for accuracy. Experience with Plant.View, PPS, MITTS, BOM, and EIM.

April 2010 – Jan. 2014, Tyson Foods, Corporate Food Safety Research Laboratory
Microbiologist

Provide a direct contact to customers and lead a group of Lab Microbiologist/Chemist direct reports. Review microbiological data and approve results before sending them to customers. Investigate root cause when a corrective action is needed. Maintain ISO documents and participate in management reviews of our quality system. Also provide consulting services to plant FSQA personnel. Trouble shoot problems with equipment. Perform tasks of a Lab Microbiologist V on an as needed basis.

Jan. 2006 - April 2010, Tyson Foods, Corporate Food Safety Research Laboratory
Lab Microbiologist IV and V

Perform general microbiological techniques that include plating and reading of PetriFilm for various organisms. Interpret selective agar plates for confirming spoilage and pathogenic bacteria isolated from meat samples. Responsible for running samples on the Bio Merieux Vidas, Bio Merieux Vitek, the Dupont Bax machines, and BioControl GDS system. Work with the Research Team to carry out various validation studies for in plant equipment and future chemical and physical interventions. Maintain and perform calibrations on equipment. Responsible for inventory.

COMPUTER SKILLS

Labware LIMS V.5, MS Word, Excel, PowerPoint, JMP, SAS

PUBLICATIONS

Handley J, Hanning I, Ricke SC, Johnson MG, Jones FT, Apple RO.2010. Temperature and Bacterial Profile of Post Chill Poultry Carcasses Stored in Processing Combo Held at Room Temperature. Journal of Food Science. 75 (8): 515-520

Handley J, Zhaohao S, Park SH, Dawoud T, Kwon YM, Ricke SC. 2015. *Salmonella* and the potential role for methods to develop microbial process indicators on chicken carcasses. In *Food Safety Emerging Issues, Technologies and Systems* (pp. 81-104) London, UK: Academic Press 2015

PROFESSIONAL MEMBERSHIP

Member of Arkansas Association of Food Protection

Figure 8. Letter of approval from Tyson Foods, Inc.

Tyson

To whom it may concern,

The Tyson Legal Department has reviewed the manuscript submitted by John Handley and may be submitted for publication to a peer reviewed journal and included in the doctoral dissertation.

Best regards

Daniel J. Zelenka, PhD.
VP Laboratory Services
Tyson Foods, Inc.