VALIDATION OF A 2% LACTIC ACID ANTIMICROBIAL RINSE AS AN ALTERNATIVE TO CHLORINE FOR MOBILE POULTRY SLAUGHTER OPERATIONS

By

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To the Faculty of Washington State University:

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VALIDATION OF A 2% LACTIC ACID ANTIMICROBIAL RINSE AS AN ALTERNATIVE TO CHLORINE FOR MOBILE POULTRY SLAUGHTER OPERATIONS

Abstract

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Poultry is a known source of foodborne pathogens; therefore, processing interventions to reduce pathogens are critical. Washington mobile poultry slaughter operators wanted to identify an antimicrobial rinse alternative to chlorine to assist with product organic labeling and increase consumer appeal. Lab and field studies were performed evaluating lactic acid as an antimicrobial rinse for poultry. The lab study examined water, chlorine (50-100ppm) and lactic acid (2%) as antimicrobial interventions to reduce *Salmonella* spp. on inoculated chicken wings. Three replications were performed. For each replication 20 inoculated wings were subjected to each treatment (no rinse, water, chlorine and lactic acid rinse) and were examined for *Salmonella* on Xylose Lysine Desoxycholate (XLD) agar. The *Salmonella* counts for lactic acid rinsed wings (0.39 $\log_{10}$ cfu/wing) were significantly ($p<0.01$) lower as compared to water rinsed (5.81 $\log_{10}$ cfu/wing) and chorine rinsed wings (5.69 $\log_{10}$ cfu/wing).

For the field study, effectiveness of lactic acid and chlorine rinses in mobile poultry slaughter operations was examined along with incidence of *Salmonella*. Two replications were performed with twenty carcasses randomly selected per treatment for each replication. Whole carcasses were sampled either immediately after evisceration, after a 3 minute 50-100ppm chlorine rinse or after a 3 minute 2% lactic acid rinse. The rinse fluid was examined for aerobic plate count (APC) on Tryptic Soy Agar (TSA) and total coliforms (TC) on Violet Red Bile Agar. Carcasses sampled immediately after evisceration were examined for the presence of *Salmonella,* and no *Salmonella* were detected. Expectedly, chlorine (3.78 $\log_{10}$ cfu/carcass) and lactic acid (2.26 $\log_{10}$ cfu/carcass) APC were significantly ($p<0.01$) lower than no rinse (4.28 $\log_{10}$ cfu/carcass), and lactic acid (2.26 $\log_{10}$ cfu/carcass) APC were significantly ($p<0.01$) lower than chlorine (3.78 $\log_{10}$ cfu/carcass). Furthermore, lactic acid TC (<0.30 $\log_{10}$ cfu/carcass estimated count below detection limit) were significantly ($p<0.01$) lower than chlorine (2.93 $\log_{10}$ cfu/carcass). Interestingly, there was no significant ($p=0.10$) difference between chlorine (2.93 $\log_{10}$ cfu/carcass) and no rinse (3.13 $\log_{10}$ cfu/carcass) treatments for TC. Clearly, both the lab and field studies validated the 2% lactic acid rinse as an alternative to 50-100ppm chlorine, providing significant reductions in Salmonella, APC and TC.

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1. Background

Poultry currently represents 30% of the total meat consumed worldwide and ranks second among muscle foods (FAO, 2006). Therefore, the microbiological safety of commercially produced poultry products is important to producers, consumers and public health officials (Okolocha and Ellerbroek, 2005; Rio et al., 2007). Excessive levels of microbial contamination are unacceptable from the standpoint of public health, storage quality, sensory quality and aesthetics of the processed product (Rio et al., 2007). Hence, reduction and control of foodborne pathogens and improving the shelf-life of poultry carcasses are critical objectives for food technologists and microbiologists (Okolocha et al., 2005).

Poultry meats are responsible for numerous foodborne illnesses and outbreaks the world over (Collins, 1997; CDC, 2000; Guard-Petter, 2001; Altekruse et al., 2006; Chittick et al., 2006). Poultry is a known source of bacterial pathogens such as *Salmonella* spp., *Campylobacter* (Bryan and Doyle, 1995), *Clostridium perfringens, Escherichia coli* (*E. coli*) O157:H7 (Cason et al., 2000) and *Listeria monocytogenes* (Chasseignauxet al., 2002). Furthermore, the various steps in poultry processing present opportunities for cross contamination with these pathogens (Anang et al., 2007). *Salmonella*, *Campylobacter* and *Clostridium perfringens* are of concern for poultry products since they are the three most frequent causes of bacterial foodborne illness in the United States (Mead et al., 1999). *Salmonella* causes an estimated 1.3 million human

foodborne illnesses and more than 500 deaths each year in the U.S (Mead et al., 1999). The USDA reports that salmonellosis costs the economy an annual estimated $2.4 billion (USDA-ERS, 1996c). In the European region, *Salmonella* serovars are reported to be responsible for 77.1% of the outbreaks of foodborne illnesses associated with poultry (W.H.O., 2001). Numerous cases of human salmonellosis have also been linked to fruits and vegetables but the most common route of contamination is through foods of animal origin, specifically poultry (Braden, 2006; Bryan et al., 1995; Todd, 1980).

Various *Salmonella* serotypes are associated with poultry meat and egg products and are capable of colonizing and infecting live chickens. Serovars such as *Salmonella* Pullorum and *Salmonella* Gallinarum tend to be host-specific for chickens whereas serovars such as *Salmonella* Typhimurium, *Salmonella* Enteritditis and *Salmonella* Heidelberg are able to infect a variety of hosts (Foley et al., 2008). The most common *Salmonella* serotypes linked with chickens in the United States are *Salmonella* Enteritditis, *Salmonella* Kentucky, *Salmonella* Heidelberg, *Salmonella* Typhimurium for clinical isolates and *Salmonella* Enteritditis, *Salmonella* Kentucky, *Salmonella* Heidelberg, *Salmonella* Typhimurium and *Salmonella* Senftenberg for non-clinical isolates (CDC, 2006).

The microbiological safety of fresh poultry carcasses is affected by a variety of factors, including the microorganisms associated with live chickens, the numbers and types of micro-organisms introduced during processing, cross-contamination, processing equipment design, efficiency of the processing methods, temperature control, and the sanitation and hygiene practices in the processing plant (Bailey et al., 1987). The detection of high levels of microorganisms indicates inadequate processing or insufficient

sanitation and hygienic practices in the plant, or both. High microbial levels suggest that the final products may contain pathogens such as *Salmonella*, *Campylobacter, Clostridium perfringens* and *Staphylococcus aureus* (Bailey et al., 1987). In a study by Bailey et al. (2002a), chicken carcasses and rinse water sampled at a slaughter plant revealed 8-34% *Salmonella* positive samples. Similarly, the study by Capita et al. (2007) to determine the prevalence of *Salmonella* in chicken carcasses in slaughterhouses in Spain showed an 18% (60 of 336 carcassess) prevalence of *Salmonella*. This prevalence percentage is comparable to the situation in most countries in recent years (Bailey et al., 2002b; Tavechio et al., 2002; Busani et al., 2005; Cardinale et al., 2005; Hernandez et al., 2005). Reiter et al. (2007) reported a prevalence of *Salmonella* at various sampling points in a poultry slaughterhouse in Brazil: transport cages, 16.7% (5 of 30); scalding water, 16.7% (5 of 30); frozen wings, 13.3% (2 of 15); frozen leg, 13.3% (2 of 15); skin of breast and leg, 10% (3 of 30).

These results indicate the need to have efficient sanitation and decontamination techniques in order to reduce cross-contamination during and after slaughter. Hence, the use of different interventions in combination with on-farm methods is essential to reduce the microbial load on the poultry carcasses (Corry et al., 2007). Several chemical interventions in the form of rinses (acids, chlorine, trisodium phosphate, ozone, hot water) have been identified to reduce surface pathogens on poultry (Anang et al., 2007; Corry et al., 2007; Bautista et al., 1997). Other intervention strategies have also been reported to reduce the microbial load on poultry including hot water, steam, steam vacuuming, bacteriophages and bacteriocins (reviewed in Hugas et al., 2008). The most common antimicrobial interventions in poultry processing include rinses and/or spray

washes of an antimicrobial solution, automated deluge and/or spray interventions of carcasses with an antimicrobial and carcass chillers where the carcasses are chilled in the presence of an antimicrobial solution (Stopforth et al., 2007). The most commonly used antimicrobial intervention in poultry processing is chlorine and typical concentrations are 20-50ppm.

2. Poultry processing -The Slaughter Operation

Commercial poultry processing involves a series of steps that transition live poultry into whole carcasses or fabricated cuts such as wings, thighs, breasts and legs. The process typically consists of stunning, bleeding, scalding, picking, and washing (Sams, 2001). A commercial processing plant is typically a highly coordinated system of mechanized or automated operations that perform all or some of the processing steps. Appendix 1 gives a simplified flow diagram for a commercial poultry slaughter line (Bolder, 1998).

Stunning is the first step in humane slaughter which renders the poultry unconscious. Stunning maybe carried out by an electrical current, gas or mechanical means. The simplest and most commonly used method of stunning is the electric shock (Sams, 2001). The systems developed for poultry are meant to render the chickens unconscious long enough to allow the neck to be cut automatically. This helps to reduce carcass damage due to slaughter-induced struggle and convulsions during bleeding. Electric stunning has also been favoured from an animal welfare perspective to minimize pain associated with the slaughter process (Sams, 2001). During stunning, the poultry are hung on shackle conveyors by their feet and their heads come in contact with a charged

saline solution (approximately 1% NaCl) such that an electrical current flows through the bird to the shackle line which serves as the ground (Sams, 2001). Stunning is accomplished by passing a sufficient amount of current through the bird for a given amount of time, to trigger a state of unconsciousness but not death. In commercial processing in the U.S., poultry pass through the stunner cabinet at a rate of 140 to 180 chickens per minute and a low electrical current of 25 to 45mA/per chicken is typically used to achieve stunning. Proper stunning results in immobilization of poultry, improved efficiency during slaughter, increased blood loss and better feather removal during picking (Sams, 2001).

Exsanguination takes place within seconds after stunning with a rotating circular blade that severs the jugular veins and carotid arteries. If the cut is too deep and severs the spinal cord, the feathers get ‘set’ as a result of nervous stimulation and makes feather removal difficult. Contrastingly, a very shallow cut results in insufficient bleeding and the residual blood causes engorged blood vessels leading to discolouration of the skin. The bird is allowed to bleed for 2-3 minutes after the neck has been cut such that approximately 30-50% of the blood is lost (Sams, 2001).

Scalding and de-feathering are the next steps. Complete feather removal can be difficult due to attachment in the follicles. Hence, the carcasses are submerged in a hot water bath (scalding) to help loosen the feathers by denaturing the proteins that hold the feathers in place. There are two typical scalding time-temperature combinations, and each produces different effects on the carcass (Sams, 2001). Scalding at 53.35˚C (128˚F) for 120 sec is called ‘soft scalding’ and loosens the feathers without causing great damage to the outer skin. This is a preferred scalding method in many parts of the world for

producing fresh poultry with a yellow skin where the yellow colour is perceived as a sign of a healthy bird. ‘Hard scalding’ is carried out at 62-64˚C (145-148˚F) for 45sec. Since this method loosens the cuticle, it is a harsher procedure than soft scalding. The advantage of hard scalding is that it facilitates feather removal to a greater extent than soft scalding conditions (Sams, 2001).

After scalding, the de-feathering process is performed by a mechanical device with many rotating, finger-like prongs, which help to detach the feathers from the carcass. The rubber ‘fingers’ rotate rapidly and rub against the carcass and the resulting abrasion pulls out the loosened feathers. Studies have been carried out where water at 50˚C maybe sprayed on the carcasses during the de-feathering process in an effort to combine scalding and de-feathering for economic reasons. However this leads to the formation of highly contaminated aerosols which maybe a possible opportunity for cross-contamination during poultry processing (Bolder, 1998). After de-feathering, the heads are pulled off the necks and along with the feathers and blood are sent to a rendering plant where they are ground and cooked into poultry fat for inclusion in animal feed. The feet are also removed at the ankle or ‘hock’ joint and are chilled (Sams, 2001).

Evisceration and inspection follow de-feathering. Evisceration involves the removal of edible and inedible viscera from the carcass. During evisceration, the intestines maybe separated from the carcass by a mechanical device. This separation reduces the possibility for cross contamination due to fecal material (Bolder, 1998). These operations are generally carried out near refrigeration temperatures. Usually, an air handling unit with cooling and heating coils is used to provide the required cooling and ventilation. Inspection of chickens is carried out to ensure that only aesthetic and

wholesome chickens, free from disease reach the market. In some countries, each carcass is required to be inspected by a qualified veterinarian, whereas in some other countries, inspection is carried out on a whole-flock basis and only selected individual carcasses are examined.

Carcasses passing inspection are thoroughly washed in an inside/outside washer. This device has many spray points that cover the outside of the carcass, and the sprayheads are positioned such that any blood clot and debris will be removed. Some processes use the inside/outside washer to subject the meat to an antimicrobial intervention such as chlorine, trisodium phosphate or organic acid. After the antimicrobial treatment the carcasses are rapidly chilled to approximately 0-4°C to preserve quality and prevent spoilage (Sams, 2001). Chilling may either be using immersion or air chilling. Both methods have been found effective but markets needs determine the choice of application. Typically, carcasses to be sold frozen are water chilled whereas non-frozen carcasses maybe either water chilled or air chilled (Bolder, 1998). In commercial poultry processing, typically, chlorine maybe incorporated in the chill water at 20-50ppm as an antimicrobial rinse to reduce bacterial levels on carcasses.

3. Use of Antimicrobial Treatments in Poultry Processing

3.1 General Overview

Antimicrobial interventions are commonly used by the industry to control food borne pathogens on poultry carcasses. Some of the routinely used treatments may be physical, chemical, biological applied alone or in combination and are usually applied before the chilling of the carcasses takes place (reviewed in Hugas et al., 2008). Several

treatments have been assessed for reducing microbial levels on the surface of meats. Treatments such as hot water and steam are being used to reduce microbial contamination on carcasses (Hugas, 2008; Sofos and Smith, 1998). In general, studies conducted on various meat types using various antimicrobial interventions such as hot water immersion, pressurized steam, acid sprays or chlorine sprays reported 1-3 $\log_{10}$ reduction of bacterial counts and a reduction in pathogen prevalence on poultry (Corry et al., 2007; Rio et al., 2007; Purnell et al., 2004; Sofos and Smith, 1998; Yang et al., 1998; Bautista et al., 1997; Morgan et al., 1996). Hot water sprays or immersion (80-85˚C), steam pasteurization or steam pressure have been described as potential applications as antimicrobial interventions for meat carcasses (reviewed in Huffman, 2002). Chemical decontamination of carcasses has been studied extensively using chlorine, acidified sodium chlorite, organic acids, peroxyacids and trisodium phosphate (reviewed in Hugas, 2008). Generally, the use of organic acids as decontaminants leads to 1-1.5 $\log_{10}$ reductions of *Salmonella* and *E. coli* O157 (Smulders and Greer, 1998). Organic acids are gaining popularity due to their efficacy in reducing the surface bacteria on meat and poultry carcasses. Lactic acid in particular has been shown to significantly reduce pathogenic bacteria on beef and pork carcasses (Hardin et al., 1995; Netten et al., 1995). Some of the above mentioned methods of decontamination have been discussed below.

3.2 Physical Decontamination Treatments

3.2.1 Hot Water Treatments

Several studies have evaluated the use of hot water as a meat decontamination technology. “The USDA-FSIS (1996a) recognizes that hot water (>74˚C) will produce a sanitizing effect on beef carcasses as illustrated by scientific evidence. The bacterial

reduction achieved by a hot water spray is caused in part by the bactericidal effect and in part by the detachment of bacteria" (Dincer and Baysal, 2004). "A 1–3 $\log_{10}$ reduction against spoilage bacteria and pathogenic bacteria like *Salmonella*, *E. coli* O157:H7, *Yersinia enterocolitica* and *Listeria monocytogenes* on beef and sheep carcasses can be seen by the use of hot water as an antimicrobial intervention. However, this reduction in bacterial populations depends on time as well as the temperature of contact with the hot water" (reviewed in Sofos and Smith, 1998). Immersion may be applicable to poultry since they are smaller animals, however, a large number of slaughter plants in the U.S. prefer the use of warm water to spray the carcass rather than immerse them during commercial processing (Dincer and Baysal, 2004). Further, this type of spray washing is identified as a critical control point in the slaughtering process especially for poultry carcasses. Hot water can be an effective treatment for decontamination of meat surfaces and this is an important intervention since the regulations for poultry processing vary among countries in the world. For example, "meat hygiene regulations in the European Union, do not permit any method or product decontamination wherein the product comes in contact with any other antimicrobial agent other than potable water" (Dincer and Baysal, 2004).

In a study conducted by Corry et al. (2007) a hot water immersion treatment at 75˚C for 30 sec produced a significant reduction (almost 2 $\log_{10}$) in *Campylobacter* and a 1.3 $\log_{10}$ reduction in generic *E. coli* on chicken carcasses. Purnell et al. (2004) found reductions of up to 1.71 $\log_{10}$ in aerobic plate counts, 2.17 $\log_{10}$ of Enterobacteriaceae and 1.64 $\log_{10}$ in *Campylobacter* counts in whole poultry carcass rinse samples for naturally contaminated chicken carcasses when treated at 75˚C for 30 sec. For beef

decontamination, Sofos and Smith (1998) adopted a commercial scale hot water spray cabinet developed for sheep carcasses and applied it to beef carcasses. This treatment delivered water at 20–300 kN/m^{-2} at about 90˚C, which gave a meat surface temperature of about 80˚C and 1–3 $\log_{10}$ reduction of coliforms was achieved. According to Dincer and Baysal (2004) spraying hot water at high pressures maybe applied to poultry carcasses too but it may not achieve the desired high temperatures. Further, the resulting condensate from the spray treatment can provide opportunity for growth of microorganisms in case of re-contamination. However, it can accomplish removal of visible soil. In contrast, low pressure sprays yield higher tissue temperatures but this can cause irregularly shaped carcasses or cuts (Dincer and Baysal, 2004). To summarize, the above data indicate that hot water wash applications to carcasses have been experimentally validated to reduce bacterial counts 1 to 3 $\log_{10}$ units.

3.2.2 Steam Pasteurization

Use of steam may be employed to accomplish the thermal destruction of bacteria on the surface of meat carcasses. “The USDA-FSIS (1996a) permits the use of steam for carcass decontamination. As a decontamination technique, pressurized steam has the advantage of reduced water and energy usage” (Dincer and Baysal, 2004). Advantages of steam pasteurization are that this method illustrates efficient heat transfer, lack of residues and an intense additional cleaning of the surfaces (Bolder, 1997). Furthermore, there are no regulatory constraints for an effective application of steam; however, the level of water uptake by the product and its final appearance must be taken into account (Bolder, 1997). Although steam pasteurization has been successfully commercialized and are in use in many large beef slaughter facilities in the United States, the success of the

application in poultry depends upon the time of exposure and steam penetration which could affect the appearance of meat (reviewed in Huffman, 2002; Sofos and Smith, 1998). Exposure to steam (105˚C) for beef carcasses usually lasts for 6-8sec. With regard to poultry, reduction of surface bacteria using steam without cooking the underlying meat is possible (Morgan et al., 1996). "Morgan et al. (1996) described an experimental device for carcass treatment with superheated steam (126-139°C) with an exposure time of 52-124msec. This system resulted in a reduction of 3 $\log_{10}$ cycles of *L. innocua* counts on poultry". However, there are some limitations with the use of steam pasteurization. This method poses a difficulty in application in a continuous production process. Also, there is an extremely short application time due to carcass damage (Bolder, 1997). Hence this can be an intermediate meat decontamination technique that further aids carcass contamination before further decontamination in the chilling step (Bolder, 1997; Sofos and Smith, 1998).

3.3 Chemical Decontamination Treatments

3.3.1 Organic Acids

One of the most recently studied and now commonly used chemical decontaminants are solutions of low molecular weight organic acids (Belk, 2001). The effectiveness of organic acids can be attributed to the low pKa of organic acids (pKa of lactic acid is 3.86) therefore most of the acid molecules are present in the undissociated form. Hence, lower the pKa value, stronger is the acid. The relevance of this characteristic is that the undissociated form of an acid has higher antimicrobial activity than the dissociated form. The effect of organic acids may depend upon two factors; (1) pH, (2) the degree of dissociation of the acid (Smulders, 1995). The bactericidal effect of

organic acids is mainly attributed to the ability of the undissociated acid to penetrate the bacterial cell membrane (Bautista et al., 1997). Organic acids such as lactic acid may also cause a reduction in pH below the growth range of the microorganism. The undissociated acid molecules inhibit the metabolism of the microorganism by penetrating the bacterial cell membrane. The accumulation of the undissociated weak acid in the cytoplasm of the cell causes acidification of the cytoplasm of the microorganism and eventually leads to cell death (Booth, 1985).

Logically, some acids show more antimicrobial strength than others. Among the organic acids evaluated in literature, acetic and lactic acids have been most widely accepted as carcass decontamination rinses (reviewed in Smulders, 1995). USDA-FSIS (1996b) has approved the use of organic acid solutions such as acetic, lactic and citric acids at concentrations of 1.5-2.5%. Organic acids such as lactic acid, acetic acid, citric acid and salicylic acid, have been found effective in decreasing the number of surface microbes when applied to poultry and red meat carcasses near the end of processing (Rio et al., 2007; Hinton Jr. and Cason, 2007; Corry et al., 2007; Anang et al., 2007; Okalocha and Ellerbroek, 2005; Deumier, 2004; Fabrizio et al., 2002; Bautista et al., 1997; Netten et al., 1995; Kolsarici and Candogan 1995).

Results reported by Deumier (2004) illustrate that lactic acid was significantly more effective than citric acid at *Enterobacteriaceae* reduction on chicken legs. This is in accordance with data published by Okalocha and Ellerbroek (2005) where a 1% lactic acid treatment caused significant reductions in the aerobic plate and *Enterobacteriaceae* counts on poultry carcasses. Furthermore, the findings by Bautista et al. (1997) suggest that lactic acid significantly reduced aerobic plate counts and total coliforms on turkey

carcasses. Lactic acid as a 1.5 and 2% dip has also been shown to significantly reduce *Salmonella* counts on chicken breasts (Anang et al., 2007). Besides lactic acid, other organic acids such as salicylic acid have also been studied for their decontamination effect in poultry. *In vitro* studies by Hinton and Cason (2007) show that washing poultry skin in solutions of salicylic acid significantly reduces the number of bacteria on the skin. However, multiple washes in salicylic acid were required to achieve a significant reduction in bacterial populations.

Given that poultry have been known to harbor pathogens which are responsible for foodborne illnesses, using antimicrobial interventions to reduce these pathogens is critical. Further, an organic acid such as lactic acid may be a good antimicrobial intervention for small or mobile poultry slaughter operations where it is important to have an effective yet practical approach for pathogen reduction on poultry carcasses.

Lactic Acid

Lactic acid is of particular interest as a chemical decontamination treatment for poultry. Among the organic acids tested, it is one with great promise due to its extensively studied mechanism of inhibition and also because of its broad specificity. Lactic acid also has a GRAS (i.e. generally regarded as safe) status. Lactic acid is able to penetrate the Gram-negative bacterial cell membranes by disrupting the outer lipopolysaccharide (LPS) layer (Bautista et al., 1997). LPS molecules consist of a lipid part (lipid A) and a hydrophilic heteropolysaccharide chain protruding outward providing the cell with a hydrophilic surface (Alakomi et al., 2000). The OM acts as an efficient permeability barrier excluding macromolecules (such as bacteriocins or enzymes) and hydrophobic substances (i.e., hydrophobic antibiotics) largely due to the presence of the

lipopolysaccharide (LPS) layer on the membrane surface (Helander, 1996). The efficacy of lactic acid against Gram-negative bacteria is not surprising since lactic acid is a small water soluble molecule with affinity for the hydrophilic surface and hence can gain access to the periplasm of the microbial cell through the water-filled porin proteins of the outer membrane (OM) (Nikaido, 1996). Lactic acid has been found to be a potent OM-disintegrating agent largely attributed to the action of undissociated lactic acid molecules (Alakomi et al., 2000). Further, after undissociated lactic acid enters the cell, if the intercellular pH is higher than the pKa of the acid, the protonated acid will dissociate thus releasing a proton and hence acidifying the cytoplasm of the microorganism (Huffman, 2002).

Consequently, several researchers have evaluated the effectiveness of lactic acid as an antimicrobial agent. Bautista et al. (1997) reported that 2% lactic acid applied at 22˚C for 10 sec was an effective bactericide (2-4 $\log_{10}$ reduction in APC) reducing microbial contamination and improving the safety of poultry meat. A 1.24% lactic acid solution reduced the total aerobic bacteria on turkey carcasses by 2.4 $\log_{10}$ units. At a 4.25% lactic acid concentration, the aerobic plate counts were reduced by 4.4 $\log_{10}$ units and the coliform counts were reduced by 5.5 $\log_{10}$ units. The study concluded that the effectiveness of lactic acid is based on concentration of the solution used. The high acid concentrations were more effective than the lower concentrations. However, higher concentrations resulted in adverse sensory changes but using 1-2% lactic acid did not affect the sensory quality of turkey (Bautista et al., 1997).

Using a slightly elevated temperature along with a pressure process appears to increase the effectiveness of a lower concentration of lactic acid treatment. Yang et al.

(1998) found that at 35˚C and 413 kPa for 17 sec, a 2% lactic acid solution significantly reduced the *Salmonella* contamination on prechilled chicken carcasses by 1.7 to 2.0 $\log_{10}$ when evaluated in a poultry processing pilot plant. Similarly, a study conducted by Xiong et al. (1998) illustrated that a 2% lactic acid solution sprayed at 20˚C and 206 kPa for 30 sec brought about a 2.2 $\log_{10}$ reduction in *Salmonella* on chicken carcasses when evaluated in an experimental setting.

Applying lactic acid at different contact times during the slaughter process can alter effectiveness. For example, Izat et al. (1990) reported that broilers treated with a 1% and 2% lactic acid solutions at different stages (scald water and pre-chill or post-chill) during processing at different temperatures (57-59˚C or 0-1.1˚C) and different contact times (30sec-600sec) showed significant reductions in *Salmonella* levels. Lactic acid was more effective at reducing *Salmonella* in the pre and post chill treatments as compared to the scald water treatment. In a recent study conducted by Anang et al. (2007), dipping chicken breasts in 1.5% and 2% lactic acid solution for 10, 20 and 30 min at 25˚C caused significant reductions of 0.77-1.71 $\log_{10}$ in *Salmonella* and 0.54-2.6 $\log_{10}$ *E. coli* O157:H7 counts. In related applications with meat, lactic acid applications usually achieve a 1.0 to 2.0 $\log_{10}$ reduction in microorganisms on the surface of beef (Ransom et al., 2003). Hardin et al. (1995) reported that a beef carcass wash with water followed by a 2% lactic acid spray at 55˚C for 11 sec significantly reduced *Salmonella* and *E. coli* O157:H7. Similarly, when inoculated sheep/goat meat was sprayed with 2% lactic acid for 2-4 min at 295 kPa, Dubal et al. (2004) reported that *Salmonella* typhimurium levels were reduced below detection limits. They also reported a 0.42 $\log_{10}$ reduction in *E. coli* counts. From the above studies we see that a 1-2% lactic acid solution was successful in

reducing *Salmonella* counts by upto approximately 2 $\log_{10}$. Thus, lactic acid seems to have promising potential as a decontaminant for meat and poultry surfaces.

3.3.2 Chlorine

Chlorine has the longest history of use to control microbial contamination in poultry processing (Mead et al., 1999). Due its widespread availability, relatively low cost and efficacy and broad spectrum activity against bacteria, chlorine is the most commonly used antimicrobial agent in food processing (Tsai et al., 1992). Traditionally in meat processing, chlorinated water has been used in carcass cabinet washers, immersion chillers or equipment sprays to reduce microbial contamination (Sanders and Blackshears, 1971). Typically a 20-50ppm spray of chlorine has been employed as an antimicrobial. Its mode of action includes biosynthetic alterations in cellular metabolism and phospholipid destruction, formation of chloramines that interfere in the cellular metabolism, oxidative action with irreversible enzymatic inactivation in bacteria, and lipid and fatty acid degradation (Russell and Keener, 2007). Hypochlorous acid form of chlorine is known to be most active since it penetrates the bacterial cell wall (Lillard, 1980) and reacts with key enzymes to prevent normal respiration (Banwart, 1989). However, a major disadvantage of chlorine is its ability to bind to organic materials, rendering it ineffective in a relatively short period of time. As a result, chlorine requires constant replenishment (Lillard, 1980; Tsai et al., 1992). In addition, chlorine is known to produce chloramines, which may interfere with the chlorinated compound's activity to inactivate bacteria populations (Gelinas and Goulet, 1983). Therefore, there is a growing need to identify alternatives to chlorine due to its drawbacks.

Data published by Sanders and Blackshear (1971) illustrates that 230ppm of chlorinated water did not significantly reduce the aerobic plate counts or coliform levels on broiler carcasses. Teotia et al. (1975) reported that only a chlorine concentration as high as 300-400ppm effectively reduced *Salmonell*a from poultry carcasses. This may have been due to the loss of effectiveness from the biological or chemical chlorine demand or due to the formation of chloramines in the chlorine solution. Some poultry processing facilities have been known to use hot chlorinated water (0-50ppm at 21-54˚C) in cabinet washers to enhance removal of carcass fecal material and associated bacteria (Bashor et al., 2004).

Some researchers have found that chlorine is not an effective disinfectant for poultry carcasses. Northcutt et al. (2005) found that the counts for aerobic bacteria and *Campylobacter* were similar to the counts found on the control carcasses. Neither different temperature treatments (21.1, 43.3 or 54.4˚C) nor chlorine concentrations (0 or 50ppm) had a significant effect on aerobic plate counts, *E. coli*, *Salmonella* or *Campylobacter* levels recovered from the carcasses in this study ($p<0.05$). Conner et al. (2001) have reported that there was a tendency for an immersion chilling in cold (4˚C), chlorinated (20ppm) to reduce the microbial load as well as cross-contamination however the reductions were not statistically or practically significant. However, a more recent study by Stopforth et al. (2007) showed that a 20-50ppm spray of chlorinated water on poultry carcasses applied individually at different points along the processing line (post de-feathering, post- evisceration, inside-outside bird wash, immediately before carcass chilling, chiller exit spray or post chiller wash) reduced the aerobic counts, total

coliforms and *E. coli* by about 0.4 $\log_{10}$ while the *Salmonella* incidence was reduced by 20-25%.

3.3.3 Trisodium Phosphate

Trisodium phosphate (TSP) in a rinse or spray reduces levels of Gram-negative bacteria such as *Salmonella* spp. (Bolder, 1997). TSP is generally recognized as safe by the Food and Drug Administration and has been approved by the USDA for use as a food ingredient (USDA- FSIS, 1982) and for the reduction of *Salmonella* contamination during poultry processing (USDA-FSIS, 1994). In the United States, a TSP treatment has been patented by AvGard™, RhÔne-Poulenc, France for the removal of fat together with bacteria from the skin surfaces using an alkaline solution containing 10% TSP (Bolder, 1997). On chicken skin, Hwang and Beuchat (1995) showed that 1% TSP treatment at 4˚C for 30 min reduced the *Salmonella* counts by 1.0 to 1.6 $\log_{10}$ units. In another study conducted by Yang et al. (1998), chicken carcasses treated with 10% TSP spray at 35˚C for 17 sec at 413 kPa showed a 1.7- 2 $\log_{10}$ reduction in *Salmonella* counts and a 0.7 $\log_{10}$ reduction in aerobic plate count as compared to a control. Similarly, Rio et al. (2007) also achieved a 2 $\log_{10}$ reduction in *Salmonella* counts when inoculated poultry legs were treated (dipped) with 12% TSP for 15min (at 18˚C). These results illustrate that TSP is an effective antimicrobial; however, the mechanism of action of TSP against microorganisms is not fully understood but the bactericidal effect of TSP is attributed to its high pH, effects on the cell wall and on adherence factors of the microorganisms (Hwang and Beuchat, 1995).

3.3.4 Other

Lactoferrin

Lactoferrin, an iron binding protein has, been described as being a microbial blocking agent and thus having potential to be an antimicrobial in foods by Naidu (2000). Lactoferrin naturally occurs in milk, saliva, tears, seminal fluids, mucins and the secondary granules of neutrophils. The compound can also be extracted commercially from cheese whey or skim milk. Naidu (2000) has patented the process for production of 'activated lactoferrin' that has been awarded a generally recognized as safe (GRAS) status by the Food and Drug Administration and has been approved by the US Dept. of Agriculture for use on fresh beef to prevent bacterial contamination. Inoculation trials conducted on beef tissue show favourable results. Activated lactoferrin maybe be used as a spray treatment on fresh beef carcasses or on chilled primal cut as a microbial blocking agent that inhibits bacterial growth by interfering with adhesion/colonization, causes detachment of live microorganisms from biological surfaces, deter microbial growth and neutralize the activity of endotoxins (Naidu, 2000). The author further states that activated lactoferrin has demonstrated microbial blocking activity against a variety of food borne pathogens *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter* spp., *Vibrio* spp. and *Staphylococcus aureus*. Thus the USDA approval of this technology may potentially be a new approach to meat safety.

Pulsed Light Technology

Pulsed light is a method of food preservation that involves the use of intense and short duration pulses of broad-spectrum 'white light'. The spectrum of light for pulsed light treatment includes wavelengths in the ultraviolet (UV) to the near infrared region (380nm-750nm). A few flashes of light applied in a fraction of a second provide a high level of microbial inactivation for most applications (Dunn et al., 1991). The mode of action of the pulsed light process is attributed to the effects of the high peak power and the broad spectrum of the flash inducing photochemical or photothermal reactions in foods (Dincer and Baysal, 2004). Nucleic acids are the primary cellular target and their inactivation occurs by several mechanisms, including chemical modifications and cleavage of the DNA (Dincer and Baysal, 2004). Organisms such as *E.coli*, *S. aureus*, *B. subtilis* and *S. cerevisiae* have been inactivated using 1 to 35 pulses of light with an intensity ranging from 1–2 j per cm^2 (Dunn et al., 1995). Dunn et al., 1995 reported a 2 log_{10} unit reduction of *Listeria innocua* on hot dogs (inoculated with 3 or 5 log per wiener) after pulsed light treatment. Similarly, *Salmonella* serovars were reduced by 2 log_{10} unit on chicken wings in samples inoculated with either 5 or 2 log per cm^2 (Barbosa-Canovas, 2000). The technology for using light pulses is applicable mainly in sterilizing or reducing the microbial population on packaging or food surfaces. Light pulses may be used to reduce or eliminate the need for chemical disinfectants and preservatives (Dunn, 1996); however extensive independent research is needed to evaluate the commercial application of this technology.

4. Organic Poultry Processing

The regulations outlined in subpart-C (Production and Handling Preamble) of the National Organic Program (USDA, 2008), suggest that organic poultry production must maintain or improve the natural resources of the farm system, including soil and water quality. Producers must keep poultry and manage animal waste in such a way that supports instinctive, natural living conditions of the animal, yet does not contribute to contamination of soil or water with excessive nutrients, heavy metals, or pathogenic organisms, and optimizes nutrient recycling (National Organic Program, USDA, 2008). Living conditions for the animals must accommodate the health and natural behavior of the animal, providing access to shade, shelter, exercise areas, fresh air, and direct sunlight suitable to the animal's stage of production, or environmental conditions, while complying with the other organic production regulations.

The organic standards require that any poultry or poultry product to be sold, labeled, or represented as organic must be maintained under continuous organic management from birth or hatching until brought to market. The organic management must begin no later than the second day of life. Any portion of the feed ration that is handled must comply with organic compositional and handling requirements. The producer must not use animal drugs, including hormones, to promote growth in an animal or provide feed supplements or additives in amounts above those needed for adequate growth and health maintenance for the species at its specific stage of life. The producer must not feed animals under organic management plastic pellets for roughage or formulas containing urea or manure. Mechanical or biological methods can be used to process an agricultural product intended to be sold, labeled, or represented as '100 percent organic',

‘organic’, or ‘made with organic ingredients’ for the purpose of retarding spoilage or otherwise preparing the agricultural product for market (National Organic Program, USDA, 2008).

As per the USDA guidelines for labeling of 100% organic products, ingredients and processing aids used must be 100% organic. This poses a problem for the use of antimicrobial treatments during handling, processing or slaughter since chlorine is not approved by the USDA as an organic antimicrobial (National Organic Program, 2008). The Washington State Department of Agriculture (WSDA) has adopted the National Organic Program standards for application to organic products in Washington. Although chlorine is listed by the National Organic Program as an approved substance under ingredient or processing aids, further description states “disinfecting and sanitizing food contact surfaces, except, that, residual chlorine levels in the water shall not exceed the maximum residual disinfectant limit under the Safe Drinking Water Act (Calcium hypochlorite; Chlorine dioxide; and Sodium hypochlorite)”. While most approved non-organic substances are allowed for use as ingredients in or on organic products, the use of chlorine is specified only for disinfection of food contact surfaces. However, its use for direct contact with food surfaces is unclear. This makes the interpretation of the regulations challenging for the poultry processors who are aiming at producing and selling ‘100% organic chicken’. However, organic rules and regulations state that lactic acid is an allowed substance in or on processed products labeled as organic (WSDA, 2008; USDA, 2008). For growers and processors who wish to market a ‘100% organic chicken’, an organic antimicrobial must be used. This provided the justification for the evaluation of lactic acid as an antimicrobial for poultry in this thesis.

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Chapter 2

Manuscript

INTRODUCTION

Poultry represents approximately 30% of the world's total meat consumption (FAO, 2006). Hence, the microbiological safety of poultry products is an important concern to producers, consumers and public health officials world-wide. Poultry meat has been associated with several pathogens such as *Salmonella* spp. (Altekruse et al., 2006), *Campylobacter* spp. (Bryan and Doyle, 1995), *Escherichia coli* (*E. coli*) O157:H7 (Cason et al., 2000) and *Listeria monocytogenes* (Chasseignauxet al., 2002) that cause foodborne illnesses (Collins, 1997; CDC, 2000; Guard-Petter, 2001;; Chittick et al., 2006). *Salmonella* and *Campylobacter* are the leading causes of foodborne bacterial illnesses in the United States (Mead et al., 1999). *Salmonella* causes an estimated 1.3 million human foodborne illnesses and more than 500 deaths each year in the U.S (Mead et al., 1999). The USDA-ERS (1996) estimated that salmonellosis resulted in an approximate annual cost of $2.4 billion to the economy. In the European region, *Salmonella* serovars are reported to be responsible for 77.1% of the outbreaks of foodborne illnesses associated with poultry (W.H.O., 2001).

Several interventions have been examined either alone or in combination, to control and reduce the foodborne pathogens on poultry carcasses. Commercially used interventions include hot water, steam, chlorine, organic acids (Hugas et al., 2008; Corry et al., 2007). Biological treatments such as bacteriophages and bacteriocins have been scientifically studied for their antimicrobial potential on poultry (Atterbury et al., 2003;

Goode et al., 2003; Hugas et al., 2008). Chlorine is by far the most frequently used antimicrobial intervention due to its availability, relative low cost and efficacy (Lillard, 1980; Tsai et al., 1992). However, chlorine binds to organic materials relatively easily and becomes ineffective in a short period of time thus requiring constant replenishment (Lillard, 1980; Tsai et al., 1992). On the other hand, lactic acid has been examined as an antimicrobial intervention in poultry processing due to its thoroughly studied mechanism of action and its GRAS (generally regarded as safe) status (Anang et al., 2007; Kanellos and Burriel, 2005; Okalocha and Ellerbroek, 2005; Bautista et al., 1997). Furthermore, lactic acid has already been found to be an effective antimicrobial rinse in red meat processing (Hardin et al., 1995; Hamby et al., 1987).

Organic poultry processors in Washington were interested in identifying an alternative antimicrobial rinse to chlorine that would meet organic standards for the final product. As per the USDA guidelines for labeling of 100% organic products, ingredients and processing aids must be 100% organic (USDA- National Organic Program, 2008). The WSDA has adopted the National Organic Program standards for application to organic products in Washington (WSDA, 2008). The list of allowed and prohibited substances in the National Organic Program allows the use of chlorine for “disinfecting and sanitizing food contact surfaces, except, that, residual chlorine levels in the water shall not exceed the maximum residual disinfectant limit under the Safe Drinking Water Act (Calcium hypochlorite; Chlorine dioxide; and Sodium hypochlorite)”. This makes the interpretation of the regulations challenging for the poultry processors who are aiming at producing and selling ‘100% organic chicken’. Also, organic rules and regulations of the National Organic Program state that lactic acid is an allowed substance in or on processed

products labeled as organic (National Organic Program, 2008). Hence, there was a need to evaluate the performance of the current chlorine antimicrobial rinse versus an organic alternative, lactic acid.

Both lab as well as field studies were carried out to validate the performance of the lactic acid antimicrobial rinse for poultry. The lab inoculation study was conducted to examine water, chlorine and lactic acid as antimicrobial interventions to reduce *Salmonella spp.* on chicken surfaces. The field study was conducted to examine lactic acid and chlorine as rinses for whole chicken carcasses in mobile poultry slaughter operations. The information from this study will provide poultry processors with data regarding the ability of lactic acid to serve as an organic antimicrobial intervention to reduce microbial contamination on poultry carcasses.

MATERIALS AND METHODS

Lab Study. Chicken wings were inoculated with *Salmonella* to examine the effectiveness of water, chlorine and lactic acid rinses as antimicrobial interventions. Since the primary objective of this study was to evaluate an antimicrobial treatment that would be suitable in the field, the first step was to select a chicken cut to represent the microbial load of whole chicken carcasses. Therefore a preliminary study was conducted to determine the microbial load on chicken wings, legs, breasts and thighs (See Appendix 2). The various cuts of chicken were enumerated for background flora and spread plated on Tryptic Soy Agar (TSA, Hardy Diagnostics, Santa Maria, CA, USA) and Xylose Lysine Desoxycholate (XLD, Hardy Diagnostics, Santa Maria, CA, USA) for aerobic plate counts and hydrogen sulfide producing bacterial counts respectively. It was found

that the chicken wings had the highest microbial load when compared to legs, breasts and thighs. Hence, the wings were selected to be used in the laboratory, inoculation study. Fresh chicken wings were used for the study. The fresh chicken wings were purchased at a local grocery store and inoculated with a four strain cocktail of *Salmonella* spp. Three replications were performed.

The lab study was conducted in a manner to reflect field processing conditions and evaluate the ability of the treatments to reduce *Salmonella* levels on chicken surfaces. Although more precise methods to evaluate chlorine concentration are available, chlorine test strips are an inexpensive option for processors to monitor chlorine concentration. Therefore, chlorine test strips were used in the lab study to monitor chlorine concentration to mimic field conditions and maintain consistency of methods utilized in the lab and field studies.

Strain Activation, Cocktail and Inoculation Solution Preparation Following review of literature, four isolates of *Salmonella* spp. were utilized in this study (*Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium ST14028, *Salmonella* Heidelberg S9481 and *Salmonella* Kentucky S94611). *S. enteritidis* 13076 was acquired from ATCC. *S.* Typhimurium 14028, an ATCC strain, was acquired from the Texas Tech University, Lubbock. Isolates *S.* Heidelberg S9481 and *S.* Kentucky S94611 were poultry isolates obtained from the Washington State University Veterinary School culture collection, Washington State University, Pullman. Review of the available literature led to the choice of the four isolates used in this study. These isolates are frequently found associated with poultry and in an effort to mimic naturally occurring *Salmonella* strains in poultry these specific isolates were used in the study.

For strain activation, the frozen cultures were thawed by hand warming for approximately one minute. Then, 100µl of each of the bacterial culture was pipetted into 9ml tryptic soy broth (TSB, Hardy Diagnostics, Santa Maria, CA, USA) and incubated at 37ºC for 18-24h. On day 2, 1ml of each of the culture was inoculated into 9ml of TSB and incubated at 37ºC for 18-24h. On day 3, 1ml of each of the cultures was pipetted into 100ml of TSB in four separate 250ml media bottles. These bottles were then secured in a shaker and incubated with shaking for 18-24h at 37^{o}C.

On day 4, the cocktail and inoculation solution was prepared, and the study was performed. In an empty, sterilized 500 ml media bottle, 100ml of each of the four strains of *Salmonella* were combined using a sterilized funnel. The cocktail was mixed thoroughly by shaking at least 25 times in a 30cm arc. The inoculation solution was prepared in a 5 gallon bucket double lined with sterile biowaste bags. First, 2L of TSB were added, followed by the 400ml *Salmonella* cocktail, and the solution was mixed thoroughly using a sterilized scoopula. Then 1L of TSB was then added, and the solution was mixed thoroughly using the same scoopula. Serial dilutions of the inoculation solution were plated on Xylose Lysine Desoxycholate (XLD, Hardy Diagnostics, Santa Maria, CA, USA) to determine the concentration of the *Salmonella* cocktail.

Product Inoculation For inoculation, the selected chicken wings were gently placed three at a time in the inoculation solution for 20sec and then removed. The chicken wings were then placed on a sterile tray under a hood at room temperature for at least 20 minutes for air-drying to allow for *Salmonella* attachment (Anang et al., 2007; Xiong et al., 1998).

Antimicrobial Rinse Preparation Three antimicrobial rinses were prepared for this study: water rinse, 50-100ppm chlorine rinse and 2% lactic acid rinse. All three rinse solutions

were prepared in 5 gallon buckets lined with two sterile biowaste bags. For the water rinse, a graduated cylinder was used to measure 7.57L of tap water. Tap water was used to in order to mimic field conditions where tap or well water was used for preparing all rinse solutions. The chlorine rinse (50-100ppm) was prepared by measuring 7.57L of tap water using a graduated cylinder and mixing thoroughly with 18.75ml chlorine (Clorox® Regular Bleach, Oakland, CA, USA, with 6% sodium hypochlorite). The chlorine concentration was measured with a chlorine test strip (Chlorine test strips, LaMotte Company, Fisher Scientific), and a 50ml solution sample was collected in a sterile centrifuge tube for pH measurement. The chlorine solution concentration was initially 50-100ppm, closer to 100ppm. The average initial pH of the chlorine rise was 8.4 and average final pH measured at the end of the sampling was 7.4 (See Appendix 3 for raw data). The 2% lactic acid rinse was prepared by measuring 7.57L of tap water using a graduated cylinder and mixing thoroughly with 178.1ml of 85% lactic acid (Purac® FCC 88, Purac America, Lincolnshire, IL, USA). A 50ml solution sample was taken in a sterile centrifuge tube and the average pH of the solution was measured using a pH meter at 2.3 both before and after sampling for all three replications (See Appendix 3 for raw data).

Lab Sampling In this study, wings were randomly assigned to five treatments (n=100) and three replications were carried out. The five treatments examined were: non-inoculated, inoculated-no rinse, water rinse, chlorine rinse and lactic acid rinse. For all treatments 20 chicken wings were examined, except the inoculated-no rinse in replication one for which only 10 wings were examined. As individual wings were selected, assignment to a treatment was rotated in this order: non-inoculated, inoculated-no rinse,

water rinse, chlorine rinse and lactic acid rinse, throughout the study. For rinse treatments, each chicken wing was placed in the appropriate rinse solution for 3 minutes. For microbial sampling, wings were placed in a stomacher bag and 99 ml of 0.1% peptone water was added to the bag. Preliminary data was collected to determine an approximate weight of wings from the brand selected for the study, and during the lab study weights were recorded for wings assigned to the non-inoculated treatment (See Appendix 4). This data helped to determine that 99ml of 0.1% peptone water was appropriate for the sampling. The chicken wing was massaged by hand for 2 minutes in 99ml of 0.1% peptone water in a stomacher bag (Whirl-Pak Stomacher Bag, Fisher-Scientific). Serial dilutions were prepared and plated using the spread plate technique on XLD plates and incubated at 35˚C for 24-48h. The non-inoculated samples were plated using the spread plate technique on XLD and incubated at 35˚C for 24-48h and on TSA and incubated at 35˚C for 48h. All samples were plated in duplicate. The colonies were enumerated manually and the number of colony forming units per chicken wing (cfu/wing) was calculated.

Statistical Analysis After logarithmic transformation, data were analyzed as a completely randomized design using the mixed model procedure of SAS® (SAS Institute, Cary, NC, USA, 2003; Release 9.1). In the analysis of variance, treatment was considered a fixed effect while replication was considered a random effect. Means were separated using a least significant difference test, and differences were considered significant at $p < 0.05$.

Field Study. The following study was carried out to evaluate the effectiveness of lactic acid and chlorine antimicrobial rinses in mobile poultry slaughter operations as well as to examine the incidence of *Salmonella* on organic poultry carcasses in western

Washington. The slaughter (See Appendix 5) and sampling for the field study was carried out at poultry farms in Western Washington.

Field Sampling Two replications were performed. For each replication, carcasses were randomly assigned to three treatments with 20 carcasses assigned to each treatment. The treatments were no rinse, a 50-100 ppm chlorine rinse and a 2% lactic acid rinse. The no rinse treatment involved sampling without any antimicrobial rinse immediately after evisceration.

The chlorine rinse was 50-100ppm solution of chlorine bleach (Clorox® Regular Bleach, Oakland CA, USA, with 6% sodium hypochlorite) in water and the lactic acid was a 2% solution of 85% lactic acid (Purac® FCC 88, Purac America, Lincolnshire, IL, USA) in water. For the first replication, the chlorine rinse was measured for its chlorine level every three to four carcasses, and chlorine levels were monitored with chlorine test strips (Chlorine test strips, LaMotte Company, Fisher Scientific). The initial chlorine level was between 50-100ppm, and the chlorine levels indicated that the solution needed to be changed after the eighth, eleventh and thirteenth carcass to maintain a concentration between 50-100ppm (See Appendix 6). In the first replication, pH of the chlorine solutions was not measured. For replication two, the chlorine rinse was measured for its chlorine concentration every five carcasses. The initial chlorine level was between 50-100ppm, and chlorine levels indicated that the solution needed to be changed after the thirteenth carcass to maintain a concentration between 50-100ppm. The initial pH of the chlorine rinse was measured at 8.4. At the end of the study, the pH of the chlorine rinse was measured at 7.4 (Appendix 6).

The lactic acid rinse was a 2% solution of 85% lactic acid (Purac® FCC 88, Purac America, Lincolnshire, IL, USA) in water. During replication 1, the lactic acid solution was measured at a pH of 2.2 both before and after the study. During replication 2, the lactic acid was measured initially at 2.5 and was changed after fifteen chickens due to aesthetic reasons as the lactic acid solution showed a change in colour. The pH at the end of the sampling was measured at 2.4 (See Appendix 6).

For chlorine and lactic acid treatments, the carcasses were immersed in either chlorine or lactic acid rinse for three minutes and then sampled. A whole carcass rinse method was employed for sampling. For replication1, the entire carcass was placed in a 1 gallon Ziploc bag with approximately 200ml sterile peptone water (Becton, Dickinson and Company, Sparks, MD, USA). Prior to the study, the Ziploc bags were examined for bacterial presence (See Appendix 7). Three bags from a total of twenty four bags were randomly selected and were swabbed and then plated on Tryptic Soy Agar (TSA, Hardy Diagnostics, Santa Maria, CA, USA) for determination of aerobic plate counts and on Violet Red Bile Agar (VRBA, Hardy Diagnostics, Santa Maria, CA, USA) for total coliforms. The bags showed no bacterial growth and a few mold colonies (2 mold colonies- 1from each of two bags sampled) and hence were regarded as fit for the sampling. In replication 2, sterile poultry rinse bags were used for the whole carcass rinse.

The carcass was massaged by hand for two minutes in 200ml peptone water, and the carcass rinse was collected in a sterile 50ml centrifuge tube. The tubes were immersed in ice for at least fifteen minutes to rapidly chill to 4ºC. The carcass rinse

samples were transported to the Washington State University Food Microbiology Laboratory in Pullman, WA at 4ºC for further laboratory analysis (See Appendix 8). Microbiological analyses All samples were examined for aerobic plate counts and total coliforms and only the no rinse carcasses were examined for the incidence of *Salmonella* spp. The carcass rinses were serially diluted and plated in duplicate on Tryptic Soy Agar (TSA, Hardy Diagnostics, CA, Santa Maria, USA) for determination of aerobic plate count. Samples were also plated in duplicate on Violet Red Bile Agar (VRBA, Hardy Diagnostics, Santa Maria, CA, USA) for examination of total coliforms. Plating was performed using an automated spiral plater (Autoplate® 4000, Spiral Biotech Inc., Norwood, MA, USA). TSA plates were incubated at 35ºC for 48h and VRBA plates were incubated at 35ºC for 24h. The colonies were enumerated using an automated counting system (Q-count®, Spiral Biotech Inc., Norwood, MA, USA) and the colony forming units per carcass (cfu/carcass) was calculated.

The incidence of *Salmonella* spp. was examined for the no rinse carcasses only, and isolation procedures were slightly modified from the FDA-BAM method (2007). For *Salmonella* isolation, carcass rinses were pre-enriched in buffered peptone water (HiMedia Laboratory Inc., Mumbai, Maharashtra, India) with incubation at 37ºC for 24h. This was followed by selective enrichment with Rappaport-Vassiliadis broth (Becton, Dickinson and Company, Sparks, MD, USA) which was incubated at 42ºC for 24h and with Tetrathionate broth (Becton, Dickinson and Company, Sparks, MD, USA) which was incubated at 35ºC for 24h. After selective enrichment, samples were streaked for isolation on Xylose Lysine Desoxycholate agar (Hardy Diagnostics, Santa Maria, CA, USA) and Bismuth Sulfite agar (Hardy Diagnostics, Santa Maria, CA, USA) and

incubated at 35ºC for 24-48h. Presumptive positive colonies were examined for biochemical and serological reactions using triple sugar iron agar (Hardy Diagnostics, Santa Maria, CA, USA), lysine iron agar (Acumedia Manufacturers, Lansing, MI, USA) and a *Salmonella* latex agglutination test (Oxoid Ltd., Hampshire, England).

Statistical Analysis After logarithmic transformation, data were analyzed as a completely randomized design using the mixed model procedure of SAS® (SAS Institute, Cary, NC, USA, 2003; Release 9.1). In the analysis of variance, treatment and media type were considered fixed effects while replication was considered a random effect. Means were separated using a least significant difference test, and differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Lab Study. This inoculation study examined the ability of the water, lactic acid and chlorine rinses to reduce *Salmonella* on fresh chicken wings. Hydrogen sulfide producing bacteria (3.78 $\log_{10}$ cfu/wing) were detected on non-inoculated chicken wings and the inoculation solution had a concentration of $1x10^8$ cfu/ml. The APC levels on non-inoculated chicken wings were measured at 4.09 $\log_{10}$ cfu/wing. Inoculation with *Salmonella* significantly ($p<0.01$) increased levels of hydrogen sulfide producing bacteria levels by 2 $\log_{10}$ cycles to 5.78 $\log_{10}$ cfu/wing as shown in Figure 1. This inoculation ensured that the primary organism producing presumptive colonies on XLD were *Salmonella* and that measurable reductions were observed after the interventions were applied. *Salmonella* counts for the water rinsed wings (5.81 $\log_{10}$ cfu/wing) and the inoculated no-rinse treatment (5.78 $\log_{10}$ cfu/wing) were similar ($p=0.76$). Hence, water was not effective in reducing the level of *Salmonella* on chicken wings. These results are

supported by a study on chicken legs by Rio et al. (2007), where a water rinse treatment had no significant effect on the bacterial population (mesophiles, enterobacteriaceae, coliforms, micrococcaceae, pseudomonas, lactic acid bacteria, moulds and yeasts). An inoculation study on chicken breasts by Anang et al. (2007) investigating a sterile distilled water rinse at 25˚C for 10 min showed that it reduced the *Salmonella* counts by 0.01 $\log_{10}$ cfu/ml. When contact time with the water was increased to 20 min, the reduction in *Salmonella* was 0.05 $\log_{10}$ cfu/ml and at 30 min 0.06 $\log_{10}$ cfu/ml. This study clearly illustrates that a sterile distilled water treatment was ineffective in bringing about a significant reduction in *Salmonella* counts.

The chlorine rinsed wings (5.69 $\log_{10}$ cfu/wing) were also similar ($p=0.32$) to the inoculated no-rinse treatment (5.78 $\log_{10}$ cfu/wing). This similarity may be attributed to the dissipation of chlorine, short contact time of 3 min, and inactivation by the presence of organic matter (Lillard, 1980; Tsai et al., 1992). Northcutt et al. (2005) found that the counts for aerobic bacteria on chicken carcasses were similar to the counts found on the control carcasses. Neither different temperature treatments (21.1, 43.3 or 54.4˚C) nor chlorine concentrations (0 or 50ppm) had a significant effect on aerobic plate counts or *Salmonella* levels recovered from the carcasses in this study ($p<0.05$).

In 2002, Fabrizio et al. reported that immersion chilling of broiler carcasses in 20ppm chlorine solution at 4˚C brought about the following reductions. A significant reduction of 1.17 $\log_{10}$ cfu/ml was seen in APC when compared to an untreated control. However, for TC the 0.59 $\log_{10}$ cfu/ml reduction was not significant ($p>0.05$). Interestingly, the *Salmonella* counts increased by 0.05 $\log_{10}$ cfu/ml when the carcasses were treated with a 20ppm chlorine solution at 4˚C. This may have possibly been due to

cross-contamination while conducting the study. Stopforth et al. (2007) collected samples from 3 commercial poultry processing plants after the carcasses were treated with 20-50ppm chlorine rinse at 4˚C and found that the APC decreased by a significant 0.5 $\log_{10}$ cfu/ml and the TC by a significant 0.4 $\log_{10}$ cfu/ml. This reduction may be attributed to the low temperature at which the carcasses treated.

In the present study, the counts for chlorine rinsed wings (5.69 $\log_{10}$ cfu/wing) and the water rinsed wings (5.81 $\log_{10}$ cfu/wing) were statistically similar ($p=0.11$). This indicates that the chlorine rinse was not more effective than water. This may be attributed to the fact that chlorine not only reacts with the microorganisms but also with organic matter which creates a chlorine demand. Chlorine demand is a property that represents the capability of water is to consume chlorine is a designated time period. Also, the amount of chlorine desired for disinfection is proportional to the chlorine demand of the water (Tsai et al., 1991). Bautista et al. (1997) reported similar findings and showed that chlorine at 7.32ppm, 25ppm, 45ppm or 50ppm did not significantly ($p>0.20$) reduce *Salmonella spp.* from carcasses or the total counts when compared to a water spray. Fabrizio et al. (2002) observed that reduction in *Salmonella* Typhimurium by immersion in distilled water (0.35 $\log_{10}$ cfu/ml) and electrolyzed oxidizing (0.28 $\log_{10}$ cfu/ml) water with 20-50ppm chlorine at 4ºC were statistically similar. Another study conducted by Northcutt et al. (2005) reported that neither water rinses at (21.1, 43.3 or 54.4ºC) nor chlorine at 50ppm were found to have a significant effect on APC or *Salmonella* reductions on spray washed broiler carcasses ($p<0.05$). These studies corroborate the findings of the present research; chlorine rinse intervention was not more effective than a water rinse. However, high pressure water sprays have been illustrated to reduce aerobic

plate counts and *Salmonella* counts on poultry carcasses. Xiong et al. (1998) reported findings of a 1 $\log_{10}$ reduction in *Salmonella* counts with a 30sec water spray at 207 kPa. Also, Yang et al. (1998) found that spray washing carcasses with water for 17s at 413 kPa significantly ($p<0.05$) lowers *Salmonella* counts by 0.4 $\log_{10}$ as compared to a control. These studies clearly indicate a positive correlation between increased pressure of application of the water spray and reduction of *Salmonella* counts on the chicken carcasses.

The *Salmonella* counts for the lactic acid rinsed wings (0.39 $\log_{10}$ cfu/wing) were significantly ($p<0.01$) lower as compared to the water rinsed wings (5.81 $\log_{10}$ cfu/wing). This result agrees with data published by Anang et al. (2007) who have shown that chicken breasts dipped in a 2% lactic acid solution for 10 min brought about a 0.91 $\log_{10}$ reduction on *Salmonella enteritidis*. In a study conducted by Netten et al. (1995), a 2% lactic acid rinse solution at an elevated temperature of 37˚C, sprayed for 30-120sec resulted in a 2.9 $\log_{10}$ reduction of *Salmonella* from inoculated fresh pork carcasses, which was below the detection limits. The *Salmonella* counts for the lactic acid rinsed wings (0.39 $\log_{10}$ cfu/wing) were significantly ($p<0.01$) lower than the chorine rinsed wings (5.69 $\log_{10}$ cfu/wing). Figure 2 illustrates that in comparison to water and chlorine, lactic acid produced a significantly greater reduction in *Salmonella*.

The study illustrates clearly that chlorine was statistically similar to water rinse and the inoculated no-rinse. Also, lactic acid produced a significantly ($p<0.01$) greater reduction in *Salmonella* spp. in the lab study when compared to chlorine and water.

Field Study. No *Salmonella* were detected on the no-rinse carcasses in the study (0 of 20 carcasses). As per USDA-FSIS (2006) 16.3% of chickens, 32.4% of ground

chicken and 23.2% of ground turkey tested were contaminated with *Salmonella* in processing plants in the U.S and currently, broiler flocks in the United States are placed at an average of 19% positive for *Salmonella* (USDA-FSIS, 2006). However, since there was no incidence of *Salmonella* on the no-rinse carcasses, this indicates that the poultry used for sampling in the current study were raised and processed under sanitary and hygienic conditions.

Both chlorine and lactic acid antimicrobial treatments significantly reduced aerobic microbial population on the poultry carcasses as seen in Figure 2. Compared to the no-rinse treatment (4.28 $\log_{10}$ cfu/carcass), the chlorine rinse resulted in a small but statistically significant ($p<0.01$) 0.5 $\log_{10}$ reduction (3.78 $\log_{10}$ cfu/carcass) in aerobic plate count (APC). These results are similar to the results obtained by Bautista et al. (1997) where a 50ppm chlorine treatment at 22˚C for 10sec resulted in a reduction in both total counts and coliform counts on turkey carcasses by less than a 1 $\log_{10}$ cycle which was similar to the uninoculated control treatment.

The lactic acid rinse in this study resulted in statistically significant ($p<0.01$) 2 $\log_{10}$ reduction (2.26 $\log_{10}$ cfu/carcass) in APC in comparison with the no-rinse treatment (4.28 $\log_{10}$ cfu/carcass). Bautista et al. (1997) reported that a 1.24% lactic acid solution sprayed inside and outside the carcass at 22˚C for 10sec reduced the APC significantly by 2.4 $\log_{10}$ cycles as compared to the initial inoculation level. Further, at a lactic acid concentration of 4.25% there was a significant reduction in total coliforms by 5.5 $\log_{10}$ cycles as compared to the original inoculated controls (Bautista et al., 1997). As shown in Figure 2, the APC for the lactic acid rinsed carcasses (2.26 $\log_{10}$ cfu/carcass) in the

current study was significantly ($p<0.01$) lower than the chlorine rinsed carcasses (3.78 $\log_{10}$ cfu/carcass respectively).

For total coliforms (TC), no-rinse (3.13 $\log_{10}$ cfu/carcass) and chlorine rinsed (2.93 $\log_{10}$ cfu/carcass) carcasses were similar (Figure 2). The ability of chlorine to reduce total coliforms in the present study may have been influenced by the following factors: dissipation of the chlorine, inactivation by the presence of organic matter (Lillard, 1980; Tsai et al., 1992) and short contact time of 3min. The dissipation of chlorine is illustrated by the number of times the chlorine rinse had to be replenished during course of the study. The lactic acid rinse resulted in a significant ($p<0.01$) reduction of more than 2 $\log_{10}$ cycles (<0.30 $\log_{10}$ cfu/carcass, estimated count below detection limit) in total coliforms compared to the no-rinse treatment (3.12 $\log_{10}$ cfu/carcass) (Figure 2). This is in accordance with data published by Gulmez (2006) where there was a reduction in total coliforms when chicken wings were treated with 2% lactic acid. The counts measured after a 10min antimicrobial wash were 1.7 $\log_{10}$ cfu/ml for 2% lactic acid as compared to a distilled water rinse (3.9 $\log_{10}$ cfu/ml). However, the initial coliform counts on the chicken wings were not measured in the study. Furthermore, TC for the lactic acid rinsed carcasses (<0.30 $\log_{10}$ cfu/carcass estimated count below detection limit) were significantly ($p<0.01$) lower than the chlorine rinsed carcasses (2.93 $\log_{10}$ cfu/carcass). In our study, lactic acid resulted in a greater reduction in APC and TC than chlorine.

Summarizing, chlorine reduced the APC and TC levels by less than 1 $\log_{10}$ cfu/carcass whereas a 2% lactic acid rinse reduced both APC and TC by about 2 $\log_{10}$ cfu/carcass in the field study. Thus, lactic acid was shown to provide a significantly

greater reduction in APC and TC from the field study and *Salmonella* spp from the lab study. A 2% lactic acid rinse was shown in this study to be an effective antimicrobial intervention for field use, and its use was validated as an alternative to 50-100ppm chlorine. This makes it an attractive option for mobile poultry slaughter operations and will help with organic product labeling and increased consumer appeal.

FIGURE 1. *Salmonella* counts reported in $\log_{10}$ cfu/wing from *Salmonella* inoculated chicken wings collected after inoculation or after a water rinse, 50-100ppm chlorine rinse and 2% lactic acid rinse at ambient temperature.

[a-b] For *Salmonella* counts, treatments without a common superscript differ ($p<0.05$).

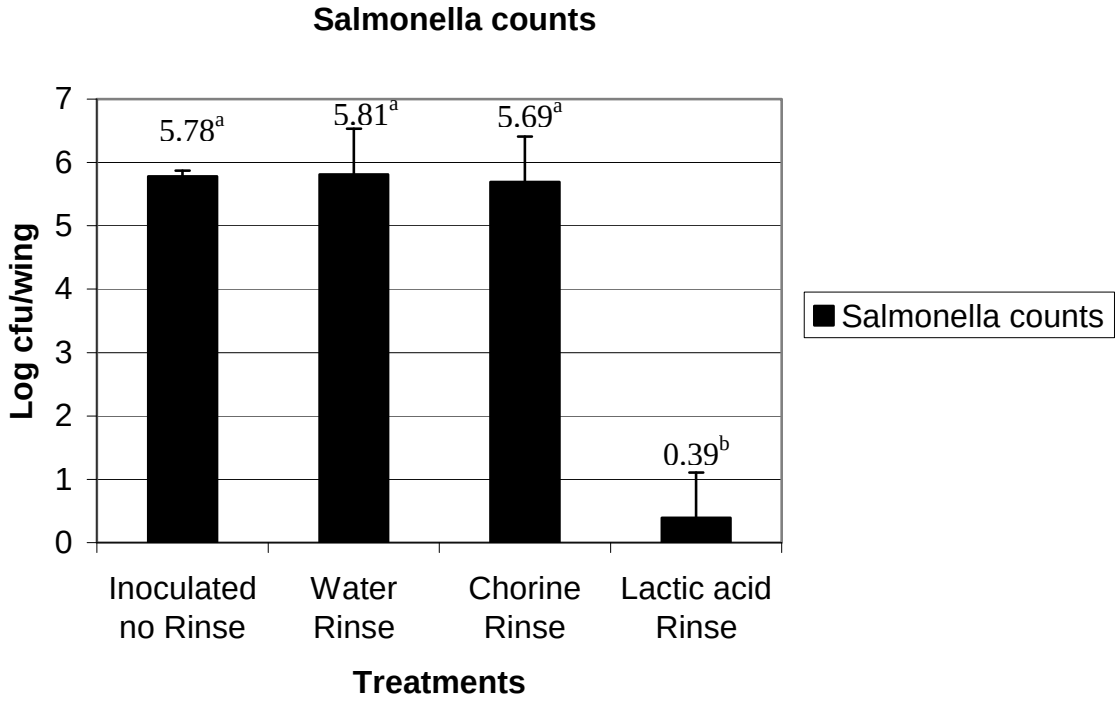
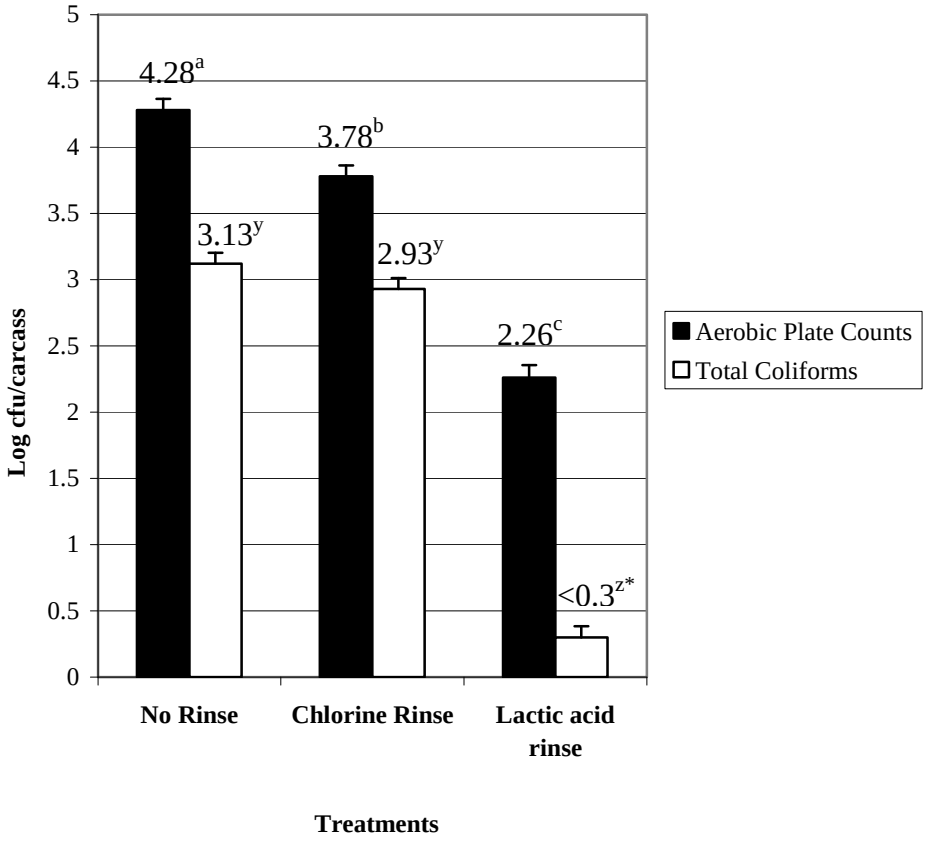


FIGURE 2: Aerobic plate counts (APC) and total coliforms (TC) reported in log10 cfu/carcass from poultry carcass rinses collected from no rinse, 50-100ppm chlorine rinse and 2% lactic acid rinse treatments at ambient temperature from two mobile slaughter operations in western Washington.

[a-c] For aerobic plate counts, treatments without a common superscript differ ($p<0.05$).
[y-z] For total coliforms, treatments without a common superscript differ ($p<0.05$).



*- <0.30 $\log_{10}$ cfu/carcass estimated count below detection limit

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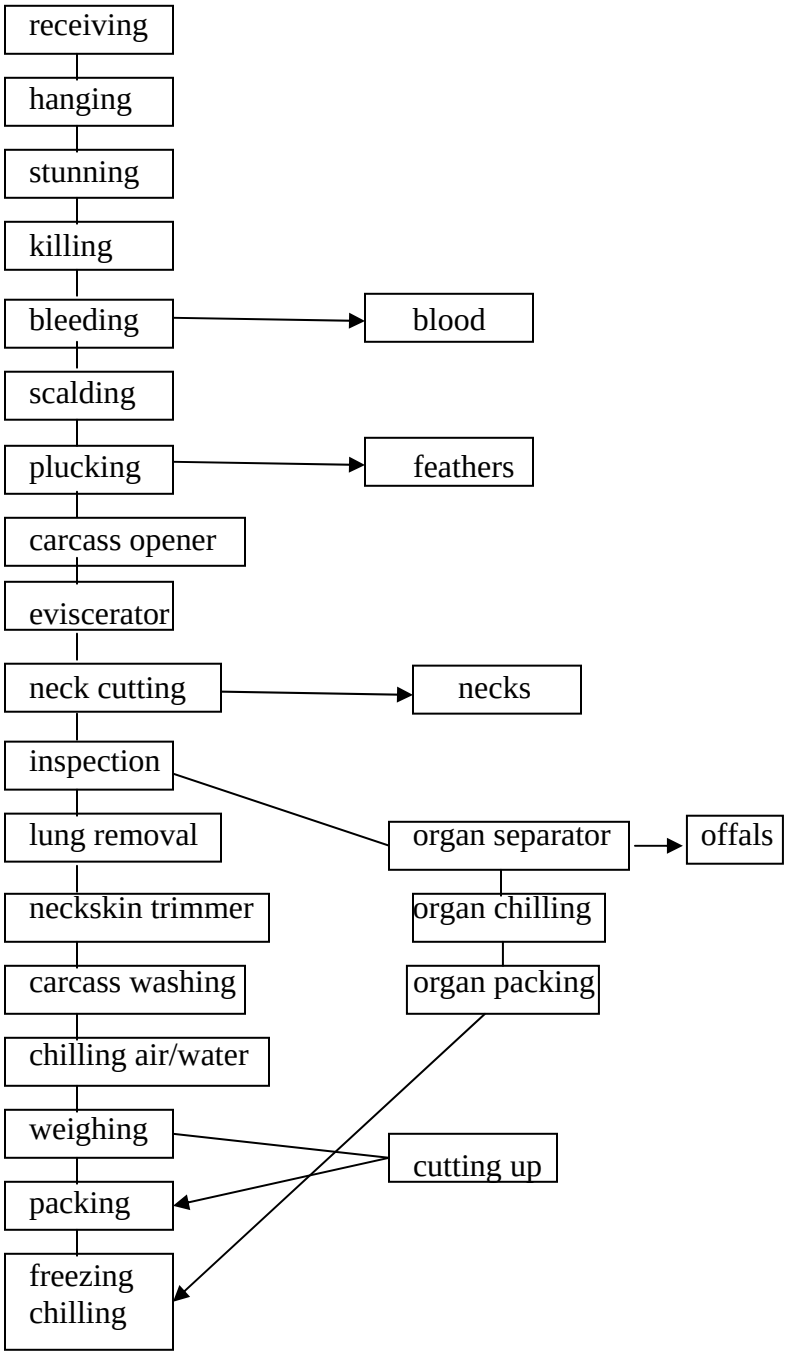
Appendix 1. Schematic flow diagram of a poultry processing line as modified from Bolder (1998)



Appendix 2. Preliminary experiment to determine the background flora on various cuts of chicken

Chicken Cut	Counts (Log_{10} cfu/ml)	
	Aerobic Plate Counts	Hydrogen sulfide producing bacterial counts
Wing	4.56	< 1 est.
Leg	2.25	< 1 est.
Thigh	3.33	< 1 est.
Breast	3.67	< 1 est.

est.- estimated count

Appendix 3. pH values of chlorine and lactic acid rinse solutions measured before and after sampling during the lab study

Treatment	pH	Replication 1	Replication 2	Replication 3
Chlorine (50-100ppm)	**Initial**	8.5	8.4	8.4
	Final	7.3	7.4	7.4
Lactic Acid (2%)	**Initial**	2.4	2.2	2.4
	Final	2.4	2.2	2.4

Appendix 4. Approximate weight of randomly selected chicken wings from preliminary and lab studies

Preliminary Study:

Wing	Weight (gms)
1	103.6
2	68.7
3	78.5
4	76.6
5	81.1
6	79.8
7	81.0
8	92.3
9	76.6
10	78.0

Average weight: 81.6 gms

Lab Study:

Replication 1

Wing	Weight (gms)
1	51.7
2	86.0
3	70.6
4	71.1
5	103.8
6	114.2
7	104.4
8	104.9
9	88.1
10	100.3

Average weight: 89.5 gms

Replication 2

Wing	Weight (gms)
1	91.1
2	76.4
3	125.4
4	72.0
5	104.3
6	90.9
7	132.8
8	96.3
9	103.1
10	72.4
11	84.4
12	111.4
13	124.7
14	121.6
15	99.7
16	115.8
17	139.2
18	144.7
19	94.3
20	131.5

Average weight: 106.6 gms

Replication 3

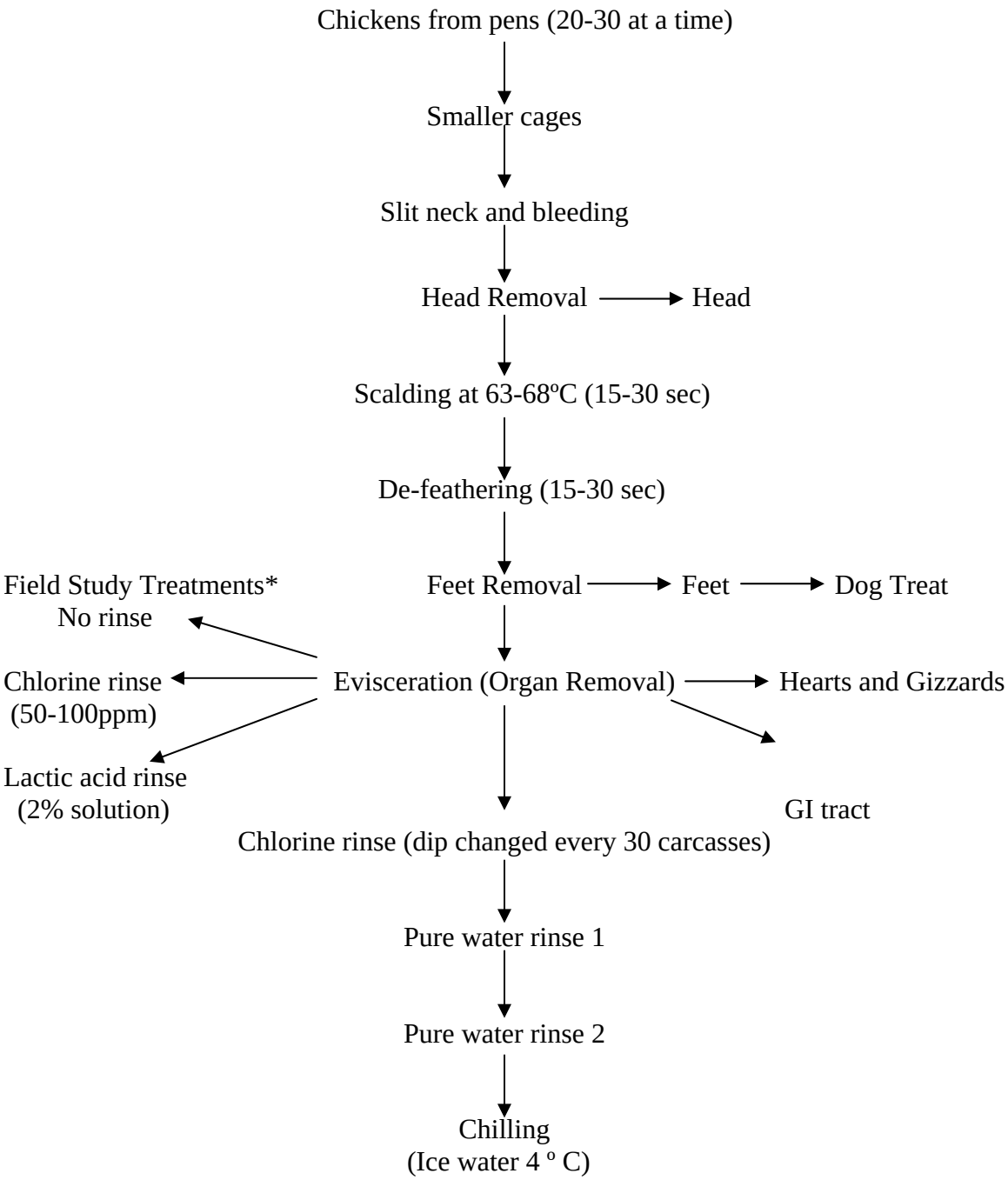
Wing	Weight (gms)
1	147.9
2	126.5
3	121.9
4	104.5
5	96.1
6	125.7
7	122.7
8	99.0
9	101.0
10	106.3
11	104.0
12	102.4
13	92.3
14	95.0
15	110.4
16	98.2
17	100.9
18	74.5
19	95.8
20	80.8

Average weight: 105.3 gms

Appendix 5. Description and schematic flow diagram of mobile poultry harvest utilized by some processors in western Washington

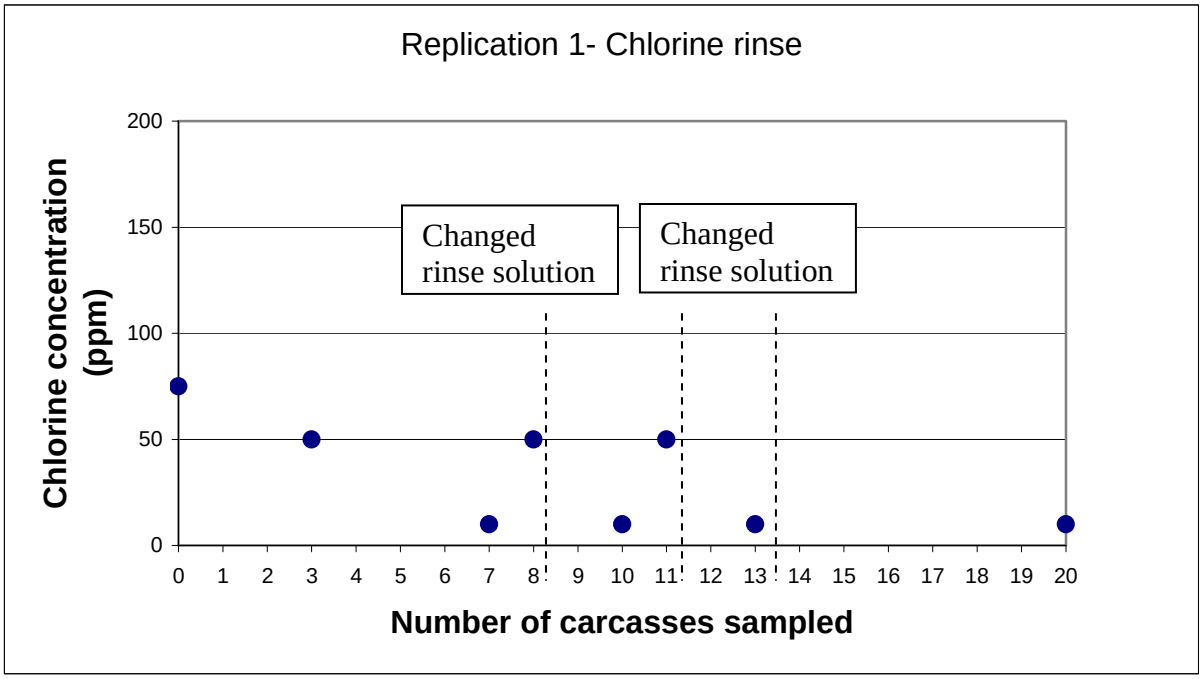
During slaughter, 20-30 chicken from the large pens (10x12x2ft) were transferred to smaller cages. The chickens were 8-10 weeks old and between 4-9lbs each. The chicken were then moved to the slaughter area two at a time, and restrained using a metal cone for exsanguination. After sufficient bleeding, the heads were removed. The next step in the slaughter process, scalding, was carried out by immersing the chicken in 63-68ºC water for 15-30sec. This greatly aids the de-feathering process as feathers are difficult to remove due to their attachment in the follicles in the skin. The hot water helps to denature the proteins that hold the feathers in place thus loosening the feathers. A de-feathering machine with finger-like rubber prongs was employed for feather removal. After de-feathering, feet removal and evisceration occurred. The removal of feet and evisceration were carried out manually and the hearts and gizzards were stored separately at approximately 4ºC until sale. Care was taken so as to not puncture the crop during evisceration in order to avoid cross contamination. The chicken carcasses were then subjected to a chlorine dip (50-100ppm). The original chlorine bleach, Clorox®, had 5.7% available chlorine. The chlorine solution was changed every 30 chicken in an effort to maintain appropriate concentration and the measured chlorine level after 30 chicken was 50ppm. The chlorine solution was followed by two pure water rinses in order to reduce residual chlorine levels on the carcasses. The carcasses were then chilled to approximately 4ºC until sale, which occurred the same day.

Flowchart for poultry harvest and field study treatments using a mobile slaughter unit in western Washington

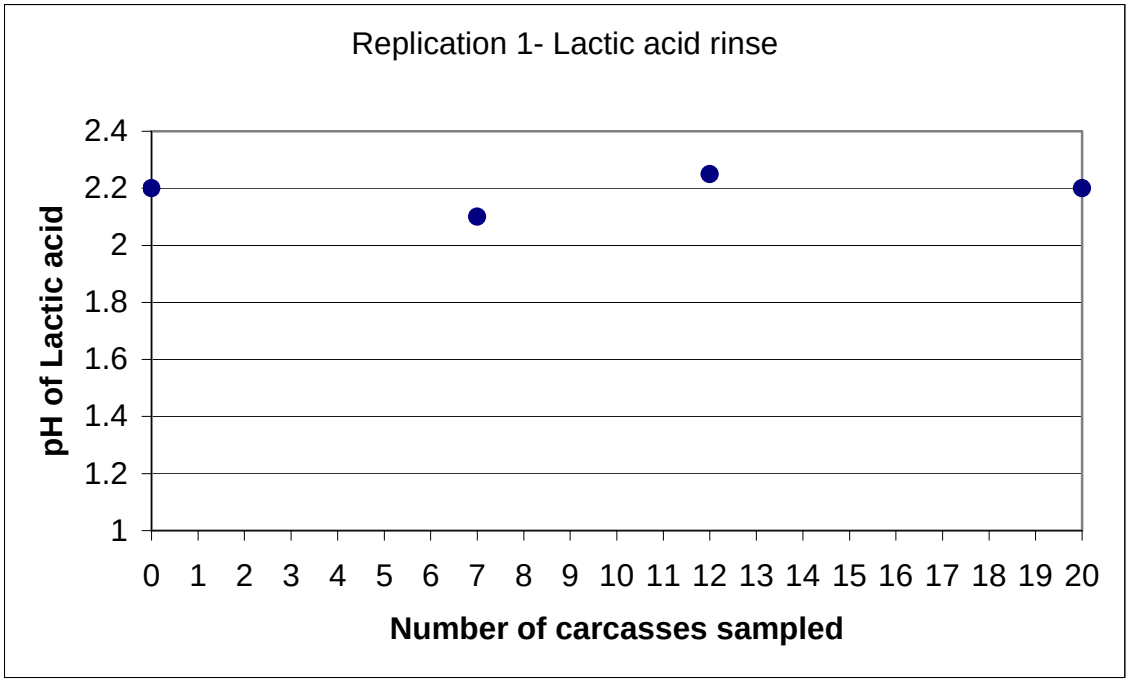


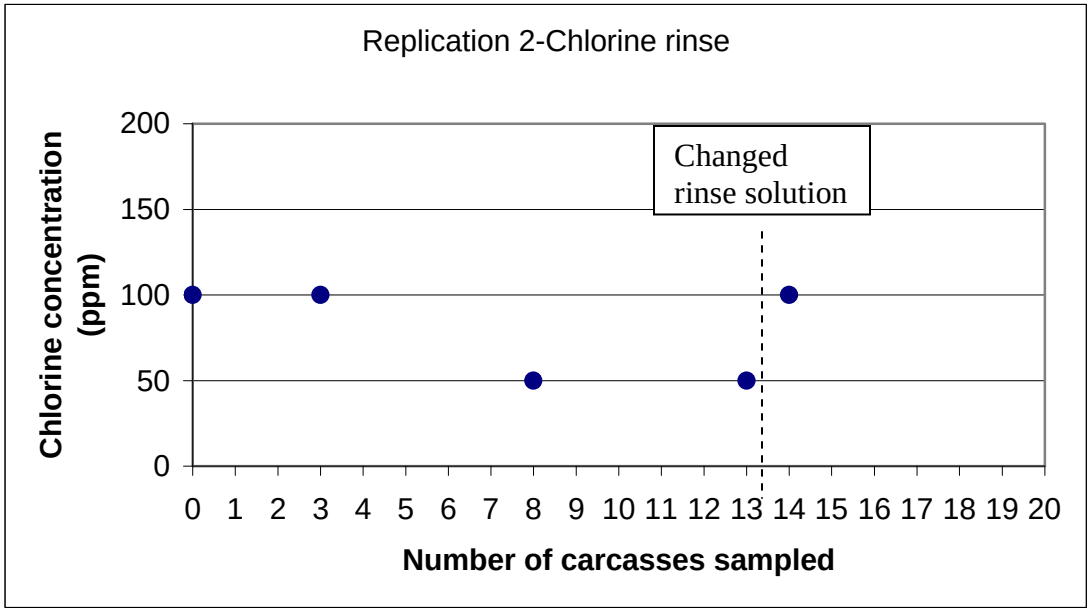
*Not part of typical harvest operations

Appendix 6. Chlorine concentration (ppm) for chlorine rinse solutions and pH values of chlorine and lactic acid rinse solutions measured at various points during sampling in the field study



Note: There was a failure to measure the concentration of fresh chlorine solution after the 13th carcass.


Replication 1- Lactic acid rinse
pH of Lactic acid
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Number of carcasses sampled

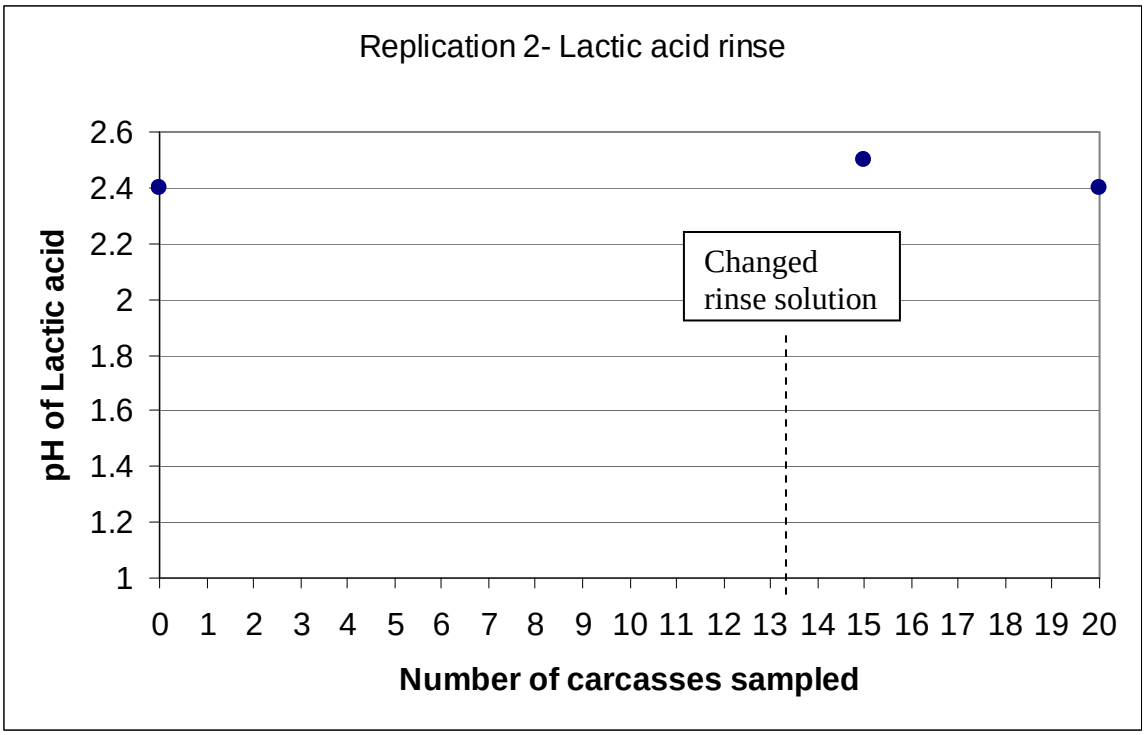


The pH values for chlorine solution in replication 2 were measured as follows

Initial (beginning of sampling): 8.4

Fresh solution after 13th carcass: 8.5

Final (end of sampling):7.4


Replication 2- Lactic acid rinse
pH of Lactic acid
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Number of carcasses sampled
Changed
rinse solution

Appendix 7. Preliminary experiment to determine the background flora on Ziploc bags to confirm ability to use during sampling in the first replication of the field study

(3 randomly selected bags of 24 bags)

Bag	Counts (Log_{10} cfu/ml)	
	Aerobic Plate Counts	Total Coliforms
1	Mold growth (1 colony-about 10mm in diameter, white colour with sponge like appearance)	< 1 est.
2	Spreader	< 1 est.
3	Mold growth(1 colony-about 10mm in diameter, white colour with sponge like appearance)	< 1 est.

est.- estimated count

Appendix 8. Preliminary chilling experiment to determine the temperature decline and temperature of samples stored and transported in a cooler

In order to determine the amount of time for rapid chilling of samples and ability to maintain storage temperature during transport, the following experiment was carried out. Centrifuge tubes were filled with approximately 50 ml of water and placed on ice in the coolers. Only one layer of tubes was placed in the cooler and no tubes were allowed to be completely immersed in the ice. The tubes were allowed to chill for 10 minutes then 5 tubes were measured randomly for temperature every 5 minutes until sample temperature seemed to stabilize. Once the temperature stabilized, test tubes were placed in racks and the ice from the coolers was removed. Ice packs were placed on the bottom of the cooler to cover the bottom and the centrifuge tubes racks were positioned in the cooler and the temperature was monitored every half hour for 5 hours.

It was determined that tubes needed to be immersed in ice for at least 15 minutes to prevent additional microbial growth, and this temperature could be maintained during transportation.

Replication 1

Time (min)	Temperature (˚C)				
	$Tube_1$	$Tube_2$	$Tube_3$	$Tube_4$	$Tube_5$
0	19.3	19.8	19.0	19.6	19.8
10	4.1	4.8	3.8	2.9	2.5
15	1.9	1.8	1.5	2.1	2.2
20	0.6	1.2	2.0	0.2	0.6
25	-0.7	-1.2	-1.2	-1.1	-1.0
30	-1.6	-1.6	-1.9	-1.6	-2.6
35	-2.9	0.2	0.2	0.1	0.1
40	0.2	-3.7	0.2	0.2	-4.5
45	0.2	0.3	0.2	0.2	0.2
50	0.2	0.2	0.1	0.2	0.2
55	0.3	0.2	0.2	0.3	0.1
60	0.3	0.2	0.2	0.2	0.1

After temperature stabilized:

Time (min)	Temperature (˚C)				
	$Tube_1$	$Tube_2$	$Tube_3$	$Tube_4$	$Tube_5$
30	0.2	0.2	0.1	0.2	0.1
60	0.1	0.1	0.2	0.1	0.2
90	0.2	0.2	0.2	0.2	0.2
120	0.2	0.2	0.2	0.2	0.2
150	0.2	0.2	0.2	0.2	0.2
180	0.2	0.2	0.2	0.2	0.2
210	0.2	0.2	0.2	0.2	0.2
240	0.3	0.2	0.2	1.0	1.2
270	0.2	0.6	0.7	0.5	0.8
300	1.6	1.8	0.9	0.2	0.4

Replication 2

Time (min)	Temperature (˚C)				
	$Tube_1$	$Tube_2$	$Tube_3$	$Tube_4$	$Tube_5$
0	20.3	20.6	20.5	20.7	20.9
10	5.0	4.7	3.4	4.7	4.0
15	2.7	3.1	3.6	3.5	3.4
20	3.6	6.0	2.8	2.9	5.6
25	4.7	3.1	5.0	2.9	2.7
30	2.3	2.4	2.3	2.6	2.8

After temperature stabilized:

Time (min)	Temperature (˚C)				
	$Tube_1$	$Tube_2$	$Tube_3$	$Tube_4$	$Tube_5$
30	2.0	3.6	3.8	3.2	2.5
60	2.8	2.5	1.2	2.6	2.5
90	1.8	2.5	1.6	1.4	3.0
120	1.3	2.6	1.7	2.3	1.6
150	2.1	1.9	1.3	2.3	2.2
180	2.0	2.5	2.1	2.4	2.5
210	2.7	2.0	2.2	1.7	2.5
240	2.8	3.3	2.9	3.0	2.3
270	3.7	3.4	2.8	3.2	3.4
300	3.3	3.5	3.7	3.6	3.4