EFFECTS OF LACTIC ACID AND COMMERCIAL CHILLING PROCESSES

ON SURVIVAL OF *SALMONELLA* SPP., *YERSINIA ENTEROCOLITICA*,

AND *CAMPYLOBACTER COLI* IN PORK VARIETY MEATS

A Thesis

by

AMANDA MARDELLE KING

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Animal Science

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Approved by:

Chair of Committee,   Margaret D. Hardin
Committee Members,   Rhonda K. Miller
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ABSTRACT

Effects of Lactic Acid and Commercial Chilling Processes on Survival of *Salmonella* spp.,

Yersinia enterocolitica, and *Campylobacter coli* in Pork Variety Meats. (August 2010)

Amanda Mardelle King, B.S., Iowa State University

Chair of Advisory Committee: Dr. Margaret D. Hardin

Current industry chilling practices with and without the application of 2% L-lactic acid were compared for their effectiveness at reducing levels of *Salmonella, Yersinia enterocolitica, Campylobacter coli,* and common indicator organisms used in industry (aerobic plate count APC, *Escherichia coli,* and coliforms) on pork variety meats. Pork livers, hearts, intestines, and stomachs were either inoculated individually with 1 of the 3 pathogens or not inoculated and subjected to 1 of 5 treatments: 1 (water wash + lactic acid spray + freeze), 2 (freeze), 3 (water wash + lactic acid spray + chill + freeze), 4 (chill + freeze), and 5 (water wash + freeze). Samples were analyzed between treatment steps and after 2 months, 4 months, and 6 months of frozen storage.

Results of effects of the steps within treatments showed that reductions in levels of pathogens after the water wash and lactic acid spray were significantly different (P<0.05) across variety meats. Treatment of variety meats with water wash and lactic acid before chilling resulted in ≥ 0.5 log CFU/sample (P<0.05) reductions when compared to chilling alone. Regardless of treatments, reductions in levels of *Salmonella* and *Y. enterocolitica* of 0.6-1.3 log CFU/sample were observed after freezing (0°C)

overnight. Freezing reduced *C. coli* by $\geq$ 2.2 log CFU/sample regardless of previous treatment.

Throughout 6 months of frozen storage, reductions were observed in levels of all microorganisms equal to or greater than 1.3 log CFU/sample. The greatest reductions were observed on samples treated with lactic acid (Treatments 1 and 3) (1.3-5.0 log CFU/sample) while the smallest reductions were reported for samples without any spray treatment (Treatments 2 and 4) (0.7-4.5 log CFU/sample). Large reductions were observed in levels of *C. coli* (2.9-5.0 log CFU/sample) for all treatments. The results of this study suggest that, while the application of a water wash followed by freezing reduced levels of pathogens by approximately 1 log CFU/sample, the application of lactic acid before chilling and freezing variety meats results in significantly larger ($P<0.05$) reductions in microorganisms. Results also show that aerobic plate counts, *E. coli,* and coliforms follow similar trends to the pathogens.

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INTRODUCTION

Despite the development of baselines and the evaluation of interventions for the decontamination of pork variety meats, refrigeration and freezing remain the most commonly used controls for microbial growth in variety meats in the pork industry. The majority of variety meats are exported and therefore processors must adhere to importing countries' approved methods for decontamination, which may or may not include an organic acid treatment. Importers of pork products cited pork safety as one of the top reasons they purchase U.S. pork *(32)*. Increased exports and the potential damage to the pork industry if an outbreak were to occur necessitate research of improved methods to decontaminate products and validate processes.

Lactic acid and chilling have both been shown to be effective in controlling pathogen growth in pork. Furthermore, Good Manufacturing Practices (GMPs) have been published for processing beef variety meats *(13)*, but there are no published guidelines for process validation for controlling pathogens in pork variety meats. In addition, the impact of the extended storage time often required for overseas shipment of variety meats on microbial levels has not been documented. Published and validated procedures for chemical decontamination in combination with chilling, freezing, and frozen storage will greatly support processors and marketers of pork variety meats.

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This thesis follows the style of *Journal of Food Protection.*

REVIEW OF LITERATURE

Characteristics of *Salmonella*

Salmonella is a Gram-negative, non-spore forming, facultatively anaerobic, rod-shaped bacteria and a member of the family Enterobacteriaceae. Approximately 2400 serovars have been identified. The optimal pH for growth is between 6.6 and 8.2, and values below 4.0 are considered bactericidal to *Salmonella (24)*. The optimum growth range is 35 to 43°C, and growth is normally limited below 7°C and above 49.5°C *(40)*. At 60°C, the D-value for *Salmonella* ranges from 2-6 min *(36)*. While the optimum water activity for *Salmonella* growth is 0.99, the minimum for *Salmonella* growth is 0.94 *(40)*. These growth parameters make *Salmonella* a concern to the meat industry as a whole, but it is also a problem in many other segments of the food industry, particularly fresh produce and food products with low water activities, such as spices. *Salmonella* is normally found in intestines of many animals, such as cattle, hogs, birds, and reptiles. However, it can be transferred to water and food through animal feces, thus becoming a risk for foodborne disease in humans.

Salmonellosis

An estimated 1.4 million cases of salmonellosis occur in the United States annually, which makes this the second leading cause of food borne illness *(31)*. The Centers for Disease Control and Prevention *(10)* reported 585 outbreaks of foodborne illness attributed to *Salmonella* in the 5 years spanning from 1998 and 2002. Of those, 17 were directly linked by epidemiological studies to pork as a vehicle of transmission.

Furthermore, other vehicles of *Salmonella* transmission included beef, eggs, dairy, poultry, vegetables, fruits and nuts, grains, and fish *(10)*. A more recent study reported 112 outbreaks associated with *Salmonella* in the United States in 2006, making it the second most common agent of foodborne disease outbreaks for that year, behind norovirus *(11)*. There have been multiple outbreaks of *Salmonella* Enteritidis associated with eggs and cross contamination from eggs to other foods that are not heat treated before consumption. For example, a large outbreak associated with milk occurred due to the fact that the milk was hauled in a trailer that had not been cleaned and had previously been hauling liquid eggs. In addition, since 1990, there have been outbreaks of multiple serovars associated with fresh tomatoes, alfalfa sprouts, cantaloupes, and undercooked ground beef *(24)*. The cause of contamination of fresh produce has been linked to cross contamination of fecal material found in the fields of fresh vegetables that do not receive a heat treatment before consumption.

In order for a case of salmonellosis to occur in a healthy adult, the person must consume approximately 10^7-10^9 cells/g *(24)*. The infectious dose most likely varies based on the sensitivity of the consumer. Twelve to 24 h after ingestion, symptoms begin including diarrhea, nausea, vomiting, and abdominal pain. In healthy adults, the disease is normally self limiting and symptoms resolve within 1 week. However, dehydration can occur and become life threatening, particularly in immunocompromised individuals, such as the elderly, infants, or individuals with cancer or HIV. Mortality rates are relatively low for the general population (4.1%), but are higher in infants and persons older than 50 (5.8% and 15%, respectively). Furthermore, a small subset of the

population, approximately 5%, can be asymptomatic carriers of *Salmonella (24)*. Proper personal hygiene, such as washing hands when working with foods, and prevention of cross contamination, such as washing a cutting board between raw products and ready-to-eat products, are methods that can help prevent cross contamination of *Salmonella.*

Characteristics of *Yersinia enterocolitica*

Yersinia enterocolitica is a Gram-negative, non-spore forming, facultatively anaerobic, rod shaped bacteria *(40)*. This organism is psychrotrophic and has been shown to grow at temperatures as low as -1.3°C and up to 42°C *(61)*. However, the optimum temperature range for growth is 30-37°C *(40)*. *Y. enterocolitica* is relatively heat sensitive and generally susceptible to thermal processes that destroy other enteric pathogens such as *Salmonella (40)*. The D-value for *Y. enterocolitica* at 60°C is approximately 0.5 min *(37)*. While growth has been observed in a pH range of 4.2-9.6, growth does not occur at pH less than 4.2 *(8, 40)*.

The different serogroups of this organism have been divided into three categories: human pathogens, animal pathogens, and environmental strains. Serogroups belonging to the human pathogen category are O:3, O:5,27, O:8, and O:9. The most widespread serogroup is O:3, which is regularly harbored in pigs, both in feces and in the oral cavity *(27)*. Other strains have been found in rodents, sheep, cattle, dogs, and cats *(40)*. *Y. enterocolitica* has also been found in lake and well water, as well as in beef, lamb, milk, and oysters, yet pork is considered the major vehicle of transmission in human illnesses association with *Y. enterocolitica (25)*.

Yersiniosis

When ingested, *Y. enterocolitica* causes gastroenteritis, usually for 5 days to 2 weeks, but it can be a cause of reactive arthritis. Sometimes, gastrointestinal symptoms persist for months after ingestion of the organism *(27)*. Mead et al. *(31)* reports that there are 96,000 cases each year in the U.S. Of the 8 outbreaks caused by *Y. enterocolitica* from 1998 to 2002, half were attributed to pork by epidemiological studies *(10)*. Most commonly, *Y. enterocolitica* is known to cause outbreaks involving chitterlings or pork intestines *(9)*. *Y. enterocolitica* and *Salmonella* have both been associated with outbreaks involving children in locations where chitterlings are prepared *(25, 62)*. Chitterlings are traditionally an ethnic dish consumed during the fall and winter holidays. Therefore, there is a seasonal pattern of yersiniosis cases, with most usually occurring during winter months. Because traditionally prepared chitterlings are thoroughly boiled, it is unlikely that consumption of the final product caused the infections, but rather cross contamination during preparation of chitterlings. Due to the risk associated with chitterlings, attempts have been made both to educate the public on preparation of variety meats and to publish GMPs for processing variety meats, although cases attributed to chitterlings do still occur.

Characteristics of *Campylobacter*

Campylobacter coli is a Gram-negative, microaerophilic, spiral-shaped bacteria found in the intestinal tract of warm blooded animals. *Campylobacter* spp. have relatively strict requirements for growth, with an optimal growth temperature of 42-43°C

and a range of 32-45°C. The optimal range of pH for growth is 6.5-7.5, and the minimum is 4.9. *Campylobacter* spp. are sensitive to lower water activity (a_w), with a minimum a_w for growth of 0.987 and an optimum a_w for growth of 0.997. The atmosphere required for growth is comprised of 5% O_2 and 10% CO_2 and 85% N_2 *(40, 53)*. In fact, 21% oxygen inhibits growth. The D-value for *Campylobacter* at 60°C is 0.2 to 0.3 min *(35)*.

Campylobacteriosis

Campylobacter spp. were first identified as human pathogens in the 1970's and are now the leading cause of food borne illness in the U.S., causing approximately 2.5 million cases each year*(31)*. Campylobacteriosis is more commonly associated with sporadic cases than in widespread outbreaks. Outbreaks have been linked to raw or improperly pasteurized milk and raw or undercooked poultry. However, the organism has also been isolated from ground beef, vegetables, and variety meats *(40)*. Ingestion of food contaminated with high levels of the organism leads to infection of the intestinal tract, causing symptoms of gastroenteritis, including watery diarrhea, which may last for for 2-7 days. The symptoms are usually self limiting, but there are multiple advanced symptoms of campylobacteriosis that can occur in immunocompromised individuals. Guillain-Barre syndrome occurs in approximately 1 out of 1000 cases, and can show symptoms such as numbness and flaccid paralysis. In about 1 out of each 100 cases of campylobacteriosis, Reiter's syndrome, also known as reactive arthritis, occurs about one week after gastrointestinal symptoms appear *(2)*.

Pork variety meats

Variety meats are edible by-products of the slaughter process, and include but are not limited to livers, hearts, thymus, chitterlings, kidneys, brains, stomachs, and tongues *(41)*. It is estimated that variety meats make up between 3 and 4% of the live weight of a market hog and the monetary value of by-products has decreased as a proportion of the live animal value over the past several years *(1)*. The majority of variety meats produced within the U.S. are exported. In 1998, Zerby et al. *(66)* reported that 60-70% of U.S. pork variety meats were exported. According to the United States Meat Export Federation, the total volume of pork variety meat exports in 2002 was approximately 176,000 metric tons, which had a corresponding value of $160 million *(56)*. The top five importing countries were Mexico, China, Canada, Japan, and Taiwan. The more recent data from January through December 2009, reported 456,000 metric tons of pork variety meats exported, with a value of $722 million *(57)*. The top countries purchasing U.S. pork variety meats in 2009 were Mexico, China, Russia, Japan, and the Association of Southeast Asian Nations (ASEAN) countries of Phillipines, Vietnam, and Singapore *(57)*. As indicated by this data, international markets for U.S. variety meats have continued to grow. To better understand foreign markets and the perceptions and expectations of U.S. products, the International Pork Quality Audit was conducted in 1994. Conclusions drawn from the interviews with importers during the audit found that customers purchase U.S. pork because of their confidence in pork safety and competitive price *(32)*. With the reported increase in exports of pork products and in order to

maintain the U.S. presence in foreign markets, it is more important than ever to maintain the safety of pork products.

Microbial contamination of variety meats

According to Swingler *(54)*, the intestines, stomachs, gut fill, hide, and feet are the largest contributors to the microbial load of carcasses. While the organs that comprise the gastrointestinal tract have a potential for high microbial loads before removal from the carcass, livers, hearts, and spleens can have low levels of contamination and a low prevalence of pathogens. Hearts are considered to be sterile *(18)*. Sheridan and Lynch *(46)* noted the evisceration table was as a source of increased contamination of variety meats. Cross contamination most likely occurred between the gastrointestinal organs and organs such as the liver and heart.

After evisceration, the process used to collect and package variety meats tends to favor microbial growth more than the process used for carcasses. Usually the entire viscera is placed onto a table, which provides opportunity for cross contamination from organs with higher microbial levels to those with lower levels, as noted by Sheridan and Lynch *(46)*. The chutes used for transferring variety meats from the evisceration area to the processing area have also been noted to be a source of contamination *(13, 66)*. Variety meats are often sorted or processed in an unchilled area adjacent to the kill floor. The room temperature is the same as the slaughter floor and is sufficient (25-30°C) for mesophilic organisms to grow *(13)*. In addition, during processing, the same worker could be handling all the variety meats and even using the same knife throughout the

process. Observers noted that in many cases, there were not areas for workers to clean and sanitize knives, aprons, and gloves *(13, 66).* Preliminary studies by Samuel et al. *(43)* found that the hands of inspectors who were working at the evisceration table tested positive for *Salmonella* over 60% of the time, and their knives were positive for nearly 20% of samples.

While some processors may use cold water or ice during packaging, packaging of products into boxes and onto pallets can take anywhere from 30 min to several hours before moving them into a cooler to begin the chilling process. During this time prior to chilling, variety meats in general provide a moist, warm (25-30°C), nutrient rich environment for the growth of microorganisms. Gill and Harrison *(19)* reported that significant growth of mesophilic organsims occurs when meat is packed at this temperature (25-30°C) before the meat is chilled below 7°C. In addition, the pH of variety meats stays relatively close to physiological pH instead of dropping to pH 5.5-5.8, as seen in carcass meats, thus maintaining an environment suitable for microbial growth *(19).*

Most studies that have looked at microbial levels of variety meats have reported results in terms of aerobic plate counts (APCs), *Escherichia coli* counts (ECC), and total coliform counts (TCC). Research by Gardner *(17)* reported that total aerobic counts on the surface of porcine livers were between 4.5 and 5 $\log_{10}/cm^2$. Similarly, Patterson and Gibbs *(38)* reported that mean initial contamination on bovine livers was 4.5-5.5 log CFU/cm^2 and on hearts was 4.6 log CFU/cm^2. On buffalo variety meats in India, initial APC levels on buffalo hearts and livers were 5.46 and 5.53 log CFU/g, respectively, and

coliform counts to be 4.98 and 5.04 $\log_{10}$ CFU/g, respectively *(45)*. Hanna et al. *(22)* showed that there were no significant differences in microbial levels when sampling different locations on the same liver, kidney, or heart. The log APC reported for pork livers ranged from 2.3-3.92, and hearts ranged from 1.48 to 4.04, which is considerably lower than other reported microbial levels.

Zerby et al. *(66)* reported APC, ECC, and TCC of boxed variety meats (cheek meat, salivary glands, tongues, livers, hearts, kidneys, stomachs, chitterlings, bungs, and front feet) before chilling, and found that in general, APCs of pork variety meats ranged from 4 to 7 log CFU/g, except for hearts, which were slightly lower. Hearts, livers, stomachs, and chitterlings had mean APC log values of 4.0-5.5, TCC of 2.0-4.2, and ECC of 1.6-4.1, with hearts consistently being lowest and chitterlings being highest within the range, suggesting that chitterlings should be targeted for decontamination treatments *(65)*. Zerby et al. *(66)* specifically noted that some of each type of variety meat had high enough levels of APCs (6-8 log CFU/g) to be considered spoiled. Woolthuis et al. *(64)* compared seasonal effects on microbial levels of APCs and reported little variation, with 6.1 log CFU/cm^2 in winter and 6.0 log CFU/cm^2 in summer on livers collected from slaughterhouses in The Netherlands. Due to the high initial microbial levels on variety meats, and those products' short inherent shelf life, improvements in processing, such as the use of decontamination treatments and accelerated chilling, can not only improve product safety, but also lengthen the shelf life.

Prevalence of pathogens in variety meats

Approximately 18% of healthy pigs have been found to be carriers of both *Salmonella* and *Y. enterocolitica,* and *C. coli* was present in 99% of pig intestines *(29)*. Similarly, 96% of fecal samples collected from pigs tested positive for *Campylobacter* spp. *(7)*. In contrast to previous data, a higher prevalence of *Salmonella* was found when organs deemed "fit for consumption" were collected from a wholesale meat market in Germany. Specifically, 45.2% of bovine variety meats (hearts, lungs, and rumens) and 63.7% of porcine variety meats (livers, hearts, lungs, esophagi) tested positive for *Salmonella*. Sinell *(47)* reported little difference in the percentage of offal pieces from which *Salmonella* was isolated in the summer and winter seasons (57.4% and 56%, respectively).

An examination of beef livers found 80% to be contaminated with *Salmonella* after the final inspection in a slaughter facility in Australia, and 30% of livers were contaminated before the viscera were placed on the evisceration table *(43)*. Research by Stern *(51)* reported that *Y. enterocolitica* was isolated from swine throats, but no *Y. enterocolitica* was isolated from bovine kidneys, tongues, or hearts. Zerby et al. *(66)* reported prevalence of *Salmonella* (15%), *Listeria monocytogenes* (16%), *Y. enterocolitica* (0%), and *C. coli* (1%), in chilled pork variety meats deemed ready for sale. However, both *Salmonella* and *L. monocytogenes* were found in a large proportion of plants sampled (100% and 80%, respectively), suggesting that prevalence is widespread *(66)*.

While *Salmonella, E. coli,* and *L. monocytogenes* are isolated from both beef and pork variety meats, *Y. enterocolitica* and *C. coli* are more commonly associated with pork. *E. coli* O157:H7 is an added concern only to the beef industry, as pigs have not been found to be reservoirs of *E. coli* O157:H7 *(63)*. *L. monocytogenes* was isolated from 15% of beef variety meats and 16% of pork variety meats *(13, 66)*. *Staphylococcus aureus* has also been found on variety meats, in 58.9% of beef and pork offals collected at a meat market in Germany *(47)*.

Lactic acid decontamination and pH effects

Due to the low relative monetary value of variety meats as compared to carcass meats, most decontamination interventions during the slaughter process are directed at carcass meats. Many studies have looked at treatments to reduce microbial contamination of carcasses and carcass meats *(6, 16, 17, 50)*. However, less research has been published on the decontamination of variety meats *(14, 39, 45, 64)*.

According to Woolthuis et al. *(64)*, contamination of variety meats in inevitable during the slaughter and evisceration process; therefore, decontamination treatments, in addition to proper Sanitation Standard Operation Procedures (SSOPs) and GMPs, provide an opportunity to improve the microbial quality of variety meat products *(13)*. Numerous studies have examined the effects of lactic acid on bacterial populations associated with beef, pork, and poultry *(48, 49, 58)*. Unlike other organic acids, lactic acid has been shown to have both an immediate bactericidal effect as well as a delayed bacteriostatic effect *(49)*.

Van Netten et al. *(58)* investigated the immediate effect of 2% lactic on *L. monocytogenes, Salmonella typhimurium,* and *C. jejuni.* When delay time (the time from application of lactic acid to the beginning of the bactericidal effects) was measured in an *in vitro* meat model system as described by Van Netten *(58)*, mean delay time decreased as pH of lactic acid decreased from pH 4.0 to 2.6 or as temperature of lactic acid increased from 21°C to 50°C. *Campylobacter* was very susceptible to treatment with 2% lactic acid (pH 2.6), even at the lowest temperature tested (21°C), with a delay time of less than 5 s, meaning that the treatment caused reductions of *Campylobacter* within 5 s of starting the treatment. When treated with lactic acid at pH 2.6 and 21°C, the delay time for *Salmonella* was 10 s; however, the delay time decreased to less than 5 s when the temperature of the lactic acid was raised to 50°C. The D-value for *Campylobacter* was less than 5 s for the 30°C, pH 2.6 lactic acid treatment, while the D-value for *Salmonella* was 28 s for the same treatment. In addition, the D-value for *Salmonella* decreased to 5 s when the temperature of the lactic acid was increased to 50°C *(57)*. Van Netten et al. *(58)* reported that mesophilic Enterobacteriaceae exhibited a delay time at pH 2.6 at 50°C of less than 5 s, suggesting that monitoring of mesophilic Enterobacteriaceae could be a good indicator of treatment effects on *Salmonella*. Anderson and Marshall *(3)* also concluded that tracking mesophilic Enterobacteriaceae is useful in indicating lactic acid effects on Gram-negative pathogens, such as *Salmonella* and *Y. enterocolitica.*

As lactic acid has become more commonly used as a carcass decontamination treatment *(3, 16, 17, 50)*, its effectiveness at reducing microbial levels on variety meats

has also been examined *(45, 49, 60, 64, 66)*. While lactic acid has been shown to have an effect on microorganisms found on meat products, the exact mode of action is not yet known. However, Gill and Newton *(21)* suggest that the main bactericidal impact is due to the drop in pH. Therefore, many researchers monitor pH when applying lactic acid.

Patterson and Gibbs *(39)* observed a 2 log CFU/cm^2 reduction of aerobic plate counts on bovine livers after immersion in 1% lactic acid (7°C) for 15 min. This was shown to increase the aerobic shelf life by 1 week *(39)*. Immersing pork livers in 0.2% lactic acid for 5 min resulted in a 2.2 log CFU/cm^2 reduction in APCs after one day vacuum packaged, refrigerated storage (4°C) and a 2.8 log CFU/cm^2 reduction after 5 days under the same storage conditions *(49)*. Woolthuis *(63)* immersed porcine livers in 0.2% lactic acid at ambient temperature for 5 min and reported a reduction in total viable counts by approximately 2 log CFU/cm^2 after 1 day of vacuum packaged storage (2.5°C) and 3 log CFU/cm^2 after 5 days of vacuum packaged storage as compared to untreated controls. The pH of livers after one day of vacuum packaged storage (2-4°C) was lower for those livers treated with lactic acid (pH 6.0) as compared to untreated controls (pH 6.3), but no difference was observed after 5 days of storage. Neither *Salmonella* nor *Yersinia* was isolated from livers treated with lactic acid, whereas both were found on untreated control livers *(64)*.

In contrast to earlier studies that used immersion to apply lactic acid *(39, 49, 64)*, Visser et al. *(60)* applied lactic acid to veal tongues using a different method, centrifugation. A 1 min centrifugation (1200 x *g*) of 10 veal tongues in 2% lactic acid (pH 2.3) resulted in reductions of nearly 3 log CFU/cm^2. The pH of the veal tongues

was measured before and after treatment with lactic acid. The pH decreased from pH

8.0 before treatment to pH 4.0 after lactic acid treatment; however, after 14 days of

storage at 3°C, there was no difference in pH based on treatments *(60)*.

Selvan et al. *(45)* studied the effect of lactic acid on buffalo offals in India.

Treatments were applied by dipping the samples (head meat, livers, hearts, and rumens)

in lactic acid (1%, 1.5%, and 2%) for 20 s, 15 s, and 10 s, respectively. After analysis of

treatments on buffalo offals, 2% lactic acid was found to be more effective in reducing

microbial levels than 1% or 1.5%, even with shorter contact time (10 s compared to 15 s

and 20 s). On fresh, chilled buffalo hearts treated with 2% lactic acid for 10 s, APC and

coliforms were significantly reduced (1.03 and 1.23 log CFU/g reductions, respectively)

as compared to controls (washed with tap water). A similar effect was observed on

fresh, chilled buffalo livers after the same treatment, with reductions of APC and

coliforms of 0.94 and 1.17 log CFU/g, respectively *(45)*.

When comparing the pH of the variety meats tested (head meat, livers, rumens,

and hearts), those samples treated with lactic acid had lower pH than untreated controls.

While the mean heart control pH was 5.81, the pH of samples treated with lactic acid

ranged from 4.74 to 5.16. The mean pH of the control livers was 6.35, and the pH of

treated samples was between 5.27 and 5.75 *(45)*. Increasing the concentration of lactic

acid from 1% to 2% (pH 2.6 and 2.4, respectively) also allowed the treatment time to be

reduced (15 s to 10 s), which would make the treatment much more practical in a

slaughter plant setting. Overall, this study concluded that treatment of buffalo variety

meats with 2% lactic acid for 10 s was more effective at reducing microbial levels than treatments using 1% (20 s) or 1.5% (15 s) lactic acid *(45)*.

Delmore et al. *(14)* treated beef variety meats with 2% lactic acid for 10 s, both by immersion (50°C) and by spraying (40-50°C, 2.4-2.7 bar). The variety meats tested were cheek meat, large intestine, lips, liver, oxtail, and tongue. Immersion in lactic acid reduced APCs by at least 0.7 log CFU/g in all 6 variety meats tested, and lactic acid spray had the same minimum reduction (0.7 log CFU/g) in 5 of the 6 variety meats. Coliform counts in all 6 variety meats tested were reduced (≥ 0.5 log CFU/g) by immersion in lactic acid, and in 5 of the 6 variety meats for the spray treatment. For reducing coliforms, immersion was significantly ($P<0.05$) more effective than spraying, with reductions of 2.1 log CFU/g after immersion and 1.2 log CFU/g after spraying large intestines, and reductions of 1.6 log CFU/g after immersion and 0.6 log CFU/g after spraying for livers. *E. coli* reductions were similar for immersion and spraying (0.9 and 0.7 log CFU/g) of all variety meats except for cheek meat and tongue, which had much lower reductions (-0.1 to 0.3 log CFU/g). Overall, the use of 2% lactic acid, applied by either immersion or spraying, reduced microbial levels on beef variety meats, thus improving the safety and quality of those products *(14)*.

Following similar procedures as used by Delmore et al. *(14)* and after developing a microbiological baseline for pork variety meats, Zerby et al. *(66)* evaluated decontamination treatments for use on pork variety meats. Decontamination treatments that were evaluated included chlorine immersion (50 ppm, 10 s, 48-50°C), hot water immersion (10 s, 75-80°C), hot water spray (10 s, 75-80°C, 35-40 psi), acetic acid

immersion (2%, 10 s, 48-50°C), acetic acid spray (2%, 10 s, 45-50°C, 35-40 psi), lactic acid immersion (2%, 10 s, 48-50°C), and lactic acid spray (2%, 10 s, 45-50°C, 35-40 psi).

In addition to measuring reductions in APC, TCC, and ECC, Zerby et al. *(66)* also measured reductions of inoculated *L. monocytogenes, Salmonella,* and *Y. enterocolitica.* Results showed that lactic acid consistently reduced APC, TCC, ECC, *Salmonella, Y. enterocolitica,* and *L. monocytogenes* on pork variety meats. The variety meats used were cheek meat, hearts, livers, salivary glands, chitterlings, stomachs, and tongues. All variety meats were treated by both spray and immersion lactic acid treatments, except for intestines, which were treated by immersion only due to the difficulty in achieving consistent surface contact with a spray treatment on the irregular surface of intestines.

On non-inoculated samples, the immersion treatment provided an equal to or greater log reduction than the spray treatment on all variety meats except for cheek meat. Reductions in APCs ranged from 0.8 to 3.3 log CFU/10 g after lactic acid spraying and 1.2 to 3.7 log CFU/10 g after immersion. A range of 1.6 to 4.3 log CFU/10 g reductions in coliforms was observed after spraying, while the reduction in coliforms after immersion was 2.5 to 4.2 log CFU/10 g. The reductions in *E. coli* counts after spraying ranged from 1.1 to 4.1 log CFU/10 g, and from 1.1 to 4.3 log CFU/10 g after immersion. As exemplified by the large ranges shown in this data, each type of variety meat responds differently to decontamination treatments *(66)*.

In the same study, rifampicin-resistant *Salmonella* and *Y. enterocolitica,* and streptomyocin-resistant *L. monocytogenes* were used to inoculate variety meats and test decontamination treatments on these products. *Salmonella* was reduced on hearts and livers by approximately 2 log CFU/g and on stomachs by 3 log CFU/g after a 10 s immersion in 2% lactic acid (48-50°C). *Salmonella* was reduced on chitterlings by about 4 log CFU/g, while *Y. enterocolitica* was reduced by approximately 6 log CFU/g. *Y. enterocolitica* was found to be very susceptible to decontamination treatments, while *L. monocytogenes* was most resistant to lactic acid treatments *(66)*. These recent studies support that 2% lactic acid treatment for 10 s applied to variety meats is effective in reducing both pathogens and general microbial contamination.

Chilling and freezing of variety meats

While there is variation across plants within the meat industry as to the use of decontamination treatments on variety meats, all variety meats are chilled or frozen before distribution. As previously discussed, a large portion of U.S. variety meats are exported, and with most variety meats being at least 3 weeks old (post mortem) by the time they reach their ultimate market *(44)*. In addition, the high initial microbial levels commonly reported for variety meats can lead to a relatively short inherent shelf life. Therefore, apart from a small percentage of products intended for local markets, variety meats are generally distributed frozen *(54)*. However, there is little guidance for processors as to chilling and freezing guidelines for variety meats. According to Savell and Pearson *(44)*, the microbial flora of variety meats and carcass meats are similarly

affected by temperature abuse and freezing. The Food Safety Inspection Service (FSIS) recommends that chilling of carcasses and variety meats begins within 1 h of exsanguination for carcasses and within 1 h of removal for variety meats. FSIS also recommends that parameters be defined, established and recorded so that carcasses reach a temperature of 4°C or less within 24 h and maintained on all products *(55)*.

Gill and Jones *(20)* evaluated commercial chilling processes of variety meats. Variation in both chilling and proliferation of *E.* coli between product units was observed, representing differences in initial temperature, box size, location on each pallet, and location in the freezer. This suggests that, particularly for livers and hearts, freezing conditions varied between individual product units, allowing for more proliferation of *E. coli (20)*. Gill and Harrison *(19)* reported that the time delay between packing and before chilling begins impacted chilling curves of variety meats. Initial temperatures ranged from 30-35°C and some products were held up to 3 h before being placed in the cooler. Products subsequently reached 5°C within 14 to 18 h *(19)*.

Most research of the microbial effects of chilling variety meats has reported an increase in microbial levels has been observed after chilling (2-10°C) *(13, 22, 39, 66)*. Delmore et al. *(13)* sampled beef variety meats at two locations within 6 beef packing plants, (A) prior to freezing and (B) after freezing, and reported increases from site A to B in APCs, ECCs and TCCs *(13)*. Hanna et al. *(22)* also observed increases in APC of beef, pork, and lamb livers, kidneys, and hearts after 5 days of storage at 2°C. In addition, freezing (-20°C) livers, kidneys, and hearts from beef, pork, and lamb for 4 days did not impact APC *(22)*. As previously discussed, the process for collecting,

packaging, and chilling variety meats often results in products sitting at ambient (25-30°C) temperature for extended periods of time before chilling begins. Hanna et al. *(22)* subjected variety meats to temperature abuse, and reported that APCs of pork livers and kidneys were affected. When livers were held at 30°C for 6 or 12 h before freezing and stored for 1 month, APCs were higher at the end of frozen storage (1.07 and 2.99 log CFU/cm^2, respectively). The same was true for kidneys, with increases of 1.38 and 3.08 log CFU/cm^2, respectively *(22)*. Patterson and Gibbs *(39)* observed higher levels of APCs on chilled beef livers, hearts, tongues, skirts, tails, and kidneys as compared to fresh variety meats (chilled ≥ 0.5 log CFU/g than fresh).

Zerby et al. *(66)* tested different decontamination treatments in a commercial pork plant setting. Pork livers, hearts, and tongues were sampled (sampling location A) after one of 6 treatments (control; 2% lactic acid spray at 50°C for 10 s; 2% lactic acid dip at 50°C for 10 s; chill at -12°C for 45 min; lactic acid spray + chill; lactic acid dip + chill). Variety meat samples for all treatments, including controls, were then placed in a freezer at 0°C for approximately 12 h followed by a blast freezer at -12°C for approximately 6 additional h before being sampled a second time (sampling location B). At site A, mean log CFU/g of APC, ECC, and TCC of all variety meats tested were lower after each of the treatments when compared to controls. The livers that did not have chilling involved in the treatment spoiled before samples were taken after freezing. However, for hearts and tongues, the mean log CFU/g of APC, ECC, and TCC increased at sampling site B in the control and the treatments without chilling. In all comparisons, chilling before freezing resulted in lower microbial levels at sampling site B. The

application of lactic acid before chilling and subsequent freezing resulted in the lowest

mean log CFU/g at site B *(66)*.

MATERIALS AND METHODS

Bacterial strains

Strains of *Salmonella* Hadar*, Y. enterocolitica,* and *C. coli* isolated from pork were obtained from the National Animal Disease Center (NADC, Ames, IA). *Salmonella* and *Y. enterocolita* were grown on Tryptic Soy Agar plates (TSA, Difco, Sparks, MD) for 24 h at 37°C. Isolated colonies were transferred to TSA slants and stored at ambient temperature as stock cultures. *C. coli* was grown on Campy Line Agar (CLA, Appendix B) at 42°C for 48 h under microaerophilic conditions. Isolated colonies were transferred to cryocare vials (Key Scientific Products, Round Rock, TX) and frozen at -80°C as stock cultures.

Isolation of rifampicin-resistant strains. To allow differentiation of *Salmonella* and *Y. enterocolitica* inoculated on samples from naturally occurring flora, rifampicin-resistant strains of *Salmonella* and *Y. enterocolitica* were selected *(28)*. A loopful of the parent organism (*Salmonella* or *Y. enterocolitica*) was inoculated into 100 ml of Tryptic Soy Broth (TSB, Difco) and incubated at 35°C for 5-8 h until absorbance read 0.8-1.2 when measured at 420 nm (Biomate 3, Thermo Electron Corporation, Waltham, MA). The tube of inoculum was centrifuged (Jouan Model MR1812, Winchester, VA) for 15 min at 4400 rpm and the supernatant was discarded. The cells were then washed in Butterfield's Buffered Phosphate (Appendix B) and again centrifuged for 15 min at 4400 rpm. This step was repeated twice. After the final wash, the supernatant was discarded, and a sterile cotton swab was used to spread the washed cells onto 10 TSA

plates containing 100 µl per liter of rifampicin (rif-TSA, Appendix B). The plates were

incubated at 35°C for 24 h. Isolated colonies were then picked up and streaked onto rif-

TSA plates and incubated under the same conditions. This step was repeated twice to

confirm rifampicin resistance. The rifampicin-resistant organisms were then transferred

to TSA slants and stored at ambient temperature for use as stock cultures.

Preliminary studies

 Evaluation of media. To differentiate the inoculum from background,

rifampicin-resistant strains of *Salmonella* and *Y. enterocolitica* were used. Rif-TSA was

used to enumerate rifampicin-resistant *Salmonella* (Appendix B). The rifampicin

solution (100 µg per liter) was prepared by dissolving 0.1 g rifampicin (Sigma-Aldrich,

St. Louis, MO) in 5 ml 99.8% methanol (Sigma-Aldrich), which was then added to 1

liter of cooled TSA. Rifampicin-Yersinia Selective Agar (Appendix B) was used to

enumerate rifampicin-resistant *Y. enterocolitica.* Ten ml rehydrated Yersinia

Antimicrobic Supplement CN (Difco) was added to each liter of cooled Yersinia

Selective Agar Base. The supplement contained 4 mg of cefsulodin and 2.5 mg of

novobiocin per liter of media. Rifampicin, prepared as described above, was added to

the media at a level of 100 µg per liter. Campy Line Agar (CLA) was used for

enumeration of *C. coli* (Appendix B).

 To compare growth on media with and without rifampicin, rif-resistant

organisms were grown in duplicate on media with and without rifampicin added.

Rifampicin-resistant *Salmonella* was grown in duplicate on TSA plates and rif-TSA

plates, and rifampicin-resistant *Y. enterocolitica* was grown in duplicate on Yersinia Selective Agar plates and rif-Yersinia Selective Agar plates. Cultures were grown in TSB for 18 h at 35°C and appropriate 10-fold dilutions were spread plated on prepoured and dried plates. All plates were incubated for 24 h at 35°C before colonies were counted.

   Growth curves. Growth rates were measured for both the parent (non resistant) and rif-resistant strains of *Salmonella* and *Y. enterocolitica* as well as for *C. coli* to determine phases of growth in order to use a culture in growth phase for preparation of inoculum. The results were also used to compare growth phases between the parent and rif-resistant strains. For *Salmonella* and *Y. enterocolitica* growth curves, 18 h cultures, grown in TSB and incubated at 35°C, were diluted to approximately 10^2 CFU/ml and 1 ml was inoculated into tubes containing 9 ml TSB and incubated at 35°C. Duplicate tubes were removed and sampled at 0, 6, 12, 16, 20, and 24 h for *Salmonella,* and 0, 6, 16, 20, 24 h for *Y. enterocolitica.* Duplicate tubes were also stored at 4°C and 0°C to observe the organisms' survival at cold temperatures. Bacteria were enumerated by spread plating 0.1 ml of the inoculum and appropriate 10-fold dilutions. TSA was used for parent *Salmonella,* rif-TSA for rif-resistant *Salmonella,* Yersinia Selective Agar for parent *Y. enterocolitica,* and rif-Yersinia Selective Agar for rif-resistant *Y. enterocolitica.* Plates were incubated at 35°C for 24 h.

   C. coli growth curves were obtained by diluting a 48 h culture to approximately 10^2 CFU/ml. Media was prepared in a series of 75 cm^2, 250 ml tissue culture flasks with canted necks and vented caps (Greiner Bio-One, Frickenhausen, Germany) by adding 50

ml blood-free Bolton Broth (containing Bolton Broth Selective Supplement (Oxoid, Hampshire, England)) and 1 Brucella-FBP agar slant (Appendix B) to each flask (personal communication, J. E. Line, Research Food Technologist, United States Department of Agriculture Agricultural Research Service). A 9 ml tube of Brucella-FBP agar was heated over a flame to melt the external part of the agar and enable it to slide into the tissue culture flask. A metal spatula was dipped in 95% ethanol and flame sterilized, and used to pry the agar slant out of the tube if needed. Following the addition of broth and agar to the flask, one loopful of *C. coli* was added and the flasks were incubated at 42°C under microaerophilic conditions. Duplicate flasks were removed and sampled at 0, 12, 24, 36, 48, 60, and 72 h. Flasks were also stored at 4°C and 0°C to measure growth at cold temperatures. *C. coli* were enumerated by plating 0.1 ml of the inoculum and appropriate 10-fold dilutions on CLA plates. Plates were incubated at 42°C under microaerophilic conditions for 48 h. Microaerophilic conditions were maintained by placing flasks (2 per bag) or plates (20 per bag) in one-gallon sized plastic bags (Ziplock, SC Johnson and Son, Inc., Racine, WI) with one GasPak EZ Campy Container System envelope (BD, Sparks, MD) (Stern, 2001).

Acid tolerance. Parent and rif-resistant strains of *Salmonella* and *Y. enterocolitica* were evaluated for resistance to 88% L-lactic acid (Birko Corporation, Henderson, CO) *(26)*. Each strain was grown for 18 h in TSB, diluted to approximately 10^2 CFU/ml, and 0.1 ml was spread plated on duplicate plates containing different levels of lactic acid at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 (Appendix B). Plates were incubated for 24 h at 35°C before counting.

Inoculation trials. To determine the method that would result in the optimal level of *Salmonella* and *Y. enterocolitica* to show reductions, two methods of inoculation (dip and drip) were tested using rif-resistant *Salmonella* and rif-resistant *Y. enterocolitica*. One loopful of the respective organism was transferred into a bottle containing 100 ml TSB and a bottle containing 250 ml TSB. The bottles containing the organisms were then grown for 18 h at 35°C and subsequently plated on rif-TSA plates and rif-Yersinia Selective Agar plates. Each inoculum (100 ml and 250 ml) was added to 6 liters of distilled water in a large, sterile plastic tub and mixed for 10 s to ensure homogeneous distribution of the microorganism. Appropriate 10-fold dilutions of the inoculum were then plated on appropriate media to determine the level of *Salmonella* or *Y. enterocolitica* in the dip.

For a second trial, fresh liver samples were obtained from hogs slaughtered at the Rosenthal Meat Science and Technology Center (RMSTC, Texas A&M University, College Station, TX) and transported in insulated containers to the Food Microbiology Laboratory (Texas A&M University, College Station, TX). Whole livers were cut into 4 pieces. In the first trial, liver pieces were submerged in the dip using 100 ml TSB for inoculum as described above (30°C) for 1 min. Pieces were then placed on a sterile steel grate to allow attachment for 10 and 30 min. Following attachment, each piece was placed in a sample bag for microbiological analysis. For the second trial, an 18 h culture of the respective organism (rif-resistant *Salmonella* or rif-resistant *Y. enterocolitica* grown in TSB) was used to inoculate pieces of pork liver. Liver pieces were placed on a tray covered in aluminum foil and pieces were inoculated by dripping 0.5 ml (10^8

CFU/ml) of the inoculum evenly over the entire surface of the sample piece. Samples were placed on a sterile steel grate for 10 and 30 min for bacterial attachment. Following attachment, each piece was placed in a sample bag for microbiological analysis. A third trial was conducted using the drip method to test attachment of *C. coli* to fresh pork liver samples. Liver samples were obtained from RMSTC and were inoculated by dripping 0.5 ml (10^8 CFU/ml) over the entire surface. Liver pieces were then placed on a piece of steel grate and held for 30 min for bacterial attachment. After the allotted attachment time, pieces were placed in sample bags for microbiological analysis.

Collection of variety meats

Samples were collected on each sample day from a small pork processor in Texas. Following slaughter and after inspection, viscera from light weight market hogs (70-90 kg) were placed in a lug and taken to a work area for removal of specific organs. The heart was removed first by cutting the blood vessels within 5 cm of the heart muscle. The heart was then cut in half and blood clots were removed. The liver was removed by cutting the bile duct, with care not to spill excess bile onto the liver surface. The lobe with the gall bladder was not used to avoid inconsistency of the liver surface. The remaining liver was cut into 6 sections per animal. The stomach was removed from the digestive tract by cutting the digestive tract within 5 cm of the stomach. To simulate industry practice, a steel rod was placed through the esophageal tube to hang the stomach and a knife was used to cut the stomach open opposite the rod to allow contents

to empty into an inedible barrel. Remaining visible digesta was removed by picking

with a gloved hand, and the stomach was cut into 4 sections. Approximately 1.2 m of

small intestine was removed from the digestive tract and sliced open to remove contents

and to expose the interior surface of the intestine. Each section was cut into 4 pieces of

approximately 30 cm in length. The sections and sample pieces were divided to be as

equal as possible in size; however, the sizes of variety meat pieces varied based on the

size of animals processed. The temperature of the variety meats when collected was

approximately 35°C. Each type of variety meat was placed into a separate plastic bag,

wrapped in shrouds, and placed in an insulated container to maintain temperature and

transported to the Texas A&M Food Microbiology Laboratory.

Preparation of inoculum

The inoculum was prepared for each sampling day by transferring one loopful of

Salmonella or *Y. enterocolitica* into each of 20-9 ml tubes of TSB and incubated for 18 h

at 35°C. The inoculum for *C. coli* was prepared for each test day by removing a bead

containing *C. coli* from the cryocare vial stored at -80°C and placed on a TSA plate

containing 5% defibrinated sheep blood (BD). The plate with the bead was incubated

under microaerophilic conditions at 42°C for 48 h. An isolated colony from the blood

plate was inoculated into the tissue culture flask system, as described above, and the

flask was incubated horizontally under microaerophilic conditions at 42°C for 48 h.

Initial wash

Upon arrival to the laboratory, a preliminary wash was applied to stomachs and intestines to remove excess digestive contents. Stomachs and intestines were placed in a lug with 12 liters of ambient temperature water (30°C) and swirled with a gloved hand for 30 s. This step was repeated once for both stomachs and intestines, after which the variety meats were returned to the insulated container in clean plastic bags, for later inoculation and treatment. After transportation to the laboratory and this washing step, the temperature of the stomachs and the intestines was approximately 30°C.

Inoculation of samples

Individual variety meat samples were removed from the insulated container and placed on aluminum foil covered trays. Based on the results of preliminary studies, each sample was inoculated by dripping 0.5 ml (10^8 CFU/ml) of the respective inoculum (*Salmonella, Y. enterocolitica,* or *C. coli*) across the surface and spread with a sterile glass rod, which resulted in counts of approximately 10^6 CFU of *Salmonella* and *Y. enterocolitica* per sample, and approximately 10^5 CFU of *C. coli* per sample. Hearts and stomachs were inoculated on the internal surface of the organ, while liver and intestines were inoculated randomly on either the internal surface or the external surface. Based on preliminary data, samples inoculated with *Salmonella* or *Y. enterocolitica* were held at ambient temperature for 10-30 min, whereas samples inoculated with *C. coli* were held 30 min to 1 h to allow bacterial attachment to the surface. Non-inoculated indicator samples (NI) of variety meats were sampled for APC, ECC, and TCC.

pH determination

On each test day, variety meat samples were monitored for pH. The initial pH of the surface of each type of variety meat was measured, and subsequently monitored throughout the treatments using a SympHony SB70P meter (VWR) and a flat bulb design SympHony electrode (VWR) by pressing the electrode bulb to the surface of each variety meat. The pH was obtained from two separate areas on each sample following each step in the treatments and averages were calculated for each variety meat at each point in the process.

Measurement of weight and surface area

Because of the natural variation in the size of the organs collected for samples, the size of each inoculated sample was measured by weight and surface area. These measurements were taken after samples were inoculated and treated with the water wash and lactic acid steps, when specified by treatment. Each sample was placed in a sample bag and weighed (Mettler Toledo Model SB16000, VWR). The surface area was measured for livers and hearts by using a 12 in. plastic ruler. Because the intestines and stomachs folded upon themselves when placed in the sample bags, 6 random samples of intestines and stomachs were measured for length and width using the ruler before bagging on each test day.

Description of spraying equipment

Two hand held polyethylene compressed air sprayers (3.8 liter RL FloMaster,
Root-Lowell Manufacturing, Lowell, MI) were used, one to apply potable water (25°C,
10 s, 50 ml) for the water wash and one to apply 2% L-lactic acid (40-50°C, 10 s, 50 ml).
A cabinet was constructed using a Rubbermaid container (117.3 liters, Rubbermaid,
Wooster, OH) , steel framework, and 4 mm clear plastic sheeting (Husky, Poly-America,
Grand Prairie, TX).

The temperature was monitored in the sprayers using a digital thermometer
(VWR Dual Thermometer, VWR) connected to Type K thermocouple sensors (Omega,
Stamford, CT). Temperature was measured by placing the thermocouple sensors in the
path of the spray to determine temperature of water and lactic acid solution at the spray
nozzle.

Description of chilling equipment

Following spray treatments, variety meat samples designated for chilling were
placed in individual sample bags (Sterile Sampling Bags, 178 x 305 mm, VWR) and
arranged in a single layer on shelves in a refrigerator (General Electric Model
TBX18HACHRWW, Louisville, KY). For samples designated to be frozen, bagged
samples were arranged in a single layer on shelves in a freezer (Kenmore Model
253.28042801, Sears Roebuck and Company, Hoffman Estates, IL). Samples stored for
frozen shelf life were held in one of three freezers (Kenmore Model 253.28042804,
Sears Roebuck and Company, Hoffman Estates, IL; General Electric Model

FCM7WMNAWW, General Electric Model FUM 21SVARWW, Louisville, KY).

Temperatures of refrigerators and freezers were monitored using Type K thermocouple

sensors attached to digital thermometers placed in the refrigerator and freezers. Sample

temperatures were monitored during initial chilling, freezing, and throughout shelf life

by inserting Type K thermocouple sensors into two samples of each type of variety meat

each test day.

Description and application of treatments

On each test day, following inoculation, samples were randomly assigned to 1 of

5 treatments. Treatment 1: water wash (WW, 25$^{\mathbf{o}}$C, 10 s, 50 ml) + lactic acid spray

(LA, 45$^{\mathbf{o}}$C, 10 s, 50 ml) + freeze (FR, 0$^{\mathbf{o}}$C), Treatment 2: freeze (FR, 0$^{\mathbf{o}}$C), Treatment

3: water wash (WW, 25$^{\mathbf{o}}$C, 10 s, 50 ml) + lactic acid spray (LA, 45$^{\mathbf{o}}$C, 10 s, 50 ml) +

chill (CH, 4$^{\mathbf{o}}$C) + freeze (FR, 0$^{\mathbf{o}}$C) , Treatment 4:   chill (CH, 4$^{\mathbf{o}}$C) + freeze (FR, 0$^{\mathbf{o}}$C),

Treatment 5: water wash (WW, 25$^{\mathbf{o}}$C, 10 s, 50 ml) + freeze (FR, 0$^{\mathbf{o}}$C). Spraying of both

the water and lactic acid was done from a distance of approximately 15 cm from the

surface of the samples. All treatments were stored for shelf life and sampled after 2

months, 4 months, and 6 months. Treatment 1, 3, and 5 samples were hung in the

cabinet with wire S-hooks made from 14 gauge galvanized steel wire. The hooks were

sanitized between samples by submersion in 95% ethanol (data not shown).

Microbiological analysis

Following treatment of each sample piece, 100 ml of 0.1% peptone water was added to each sample bag and the sample was hand massaged for 1 min. Microorganisms were enumerated by plating 1 ml (0.25 ml on each of 4 plates) of the sample rinse, 0.1 ml of the rinse, and then 0.1 ml of appropriate 10-fold dilutions of the same on prepoured and dried rif-TSA plates for *Salmonella,* Rif-Yersinia Selective Agar plates for *Y. enterocolitica,* or CLA plates for *C. coli.* Aliquots of the sample rinse were spread over the surface of the plates with a sterile bent glass rod. Rif-TSA plates and Rif-Yersinia Selective Agar plates were incubated for 24 h at 35°C before counting and reporting the number of rif-resistant organisms per sample. CLA plates were incubated microaerophilically for 48 h at 42°C, as previously described, before counting and reporting the number of organisms per sample.

Non-inoculated samples were analyzed for aerobic plate count using Aerobic Count Plate Petrifilm (3M Microbiology Products, St. Paul, MN) and total coliforms and *E. coli* using *E. coli*/Coliform Count Petrifilm (EC/CC) (3M). Counts were determined by plating 1 ml aliquots of appropriate decimal dilutions on Petrifilm plates. Plates were incubated for 48 h at 35°C for aerobic plate counts and for 24 h at 35°C for *E. coli* and total coliforms, before counting and reporting the counts per sample.

To test for background levels of rifampicin resistant *Salmonella* or *Y. enterocolitica* and naturally occurring levels of *C. coli,* 3 samples of each type of variety meat were rinsed with 100 ml of 0.1% peptone each test day. One ml of the sample rinse was plated on 4 plates of the appropriate media for the organism used for that test

day (rif-TSA, rif-Yersinia Selective Agar, or CLA). The plates were incubated and

counted to ensure that background levels of the organisms were not interfering with

tracking the inoculum level and reductions.

Statistical analysis

Microbiological data were calculated following the counting rules in the

Compendium of Methods for the Microbiological Evaluation of Foods 4th ed. (2001)

and transformed into logarithms before statistical analysis. Data were analyzed using

analysis of variance (ANOVA) and the Mixed model procedures of the Statistical

Analysis System (Version 9.2, SAS Institute, Inc., Cary, NC). Least squares means were

separated using the pdiff option when significant (P<0.05).

RESULTS

Preliminary studies

Evaluation of media. Results of the media comparison for rifampicin-resistant *Salmonella* and rifampicin-resistant *Y. enterocolitica* are shown in Table 1. While growth of *Salmonella* on TSA showed that there was 8.4 log CFU/ml in the 18 h culture, rif-TSA showed 8.5 log CFU/ml. As shown in Table 1, growth of rifampicin resistant *Y. enterocolitica* on Yersinia Selective Agar was 8.3 log CFU/ml, and growth on rif-Yersinia Selective Agar was 8.2 log CFU/ml. Like the comparison of media for enumerating *Salmonella*, this comparison shows similar growth of the organism on both types of media.

Growth curves. The comparison of growth curves between parent and rifampicin-resistant *Salmonella* and *Y. enterocolitica* showed that at 35°C, both the parent and the resistant organisms grew at similar rates, as seen in Figure 1 A and B. The variation between the rifampicin-resistant *Salmonella* strain and the parent strain in the first few hours is most likely due to variation in initial inoculum level. However, both parent and rifampicin resistant strains reached approximately 8 log CFU/ml at 12 h and became stationary from 12 to 24 h.

The rifampicin resistant strains of *Salmonella* and *Y. enterocolitica* were tested for survival at refrigeration (4°C) and freezing (0°C), and survival curves are presented in Figure 1 D and E. Initial levels for both *Salmonella* and *Y. enterocolitica* were approximately 8 log CFU/g at 0 h. Throughout 24 h storage at both 4°C and 0°C, levels

remained relatively constant for *Salmonella*. *Y. enterocolitica* stored at refrigeration temperature (4°C) stayed constant at approximately 8 log CFU/ml. However, over 24 h of frozen storage (0°C), levels of *Y. enterocolitica* declined by approximately 2 log CFU/ml.

     C. coli was grown at optimum temperature, refrigeration temperature, and freezing temperature. The results are presented in Figure 1 C and F. At 42°C under microaerophilic conditions, *C. coli* reached stationary phase at 8 log CFU/ml after 36 h of incubation. After 48 h, the inoculum level was reduced to approximtely 7 log CFU/ml and maintained that level through 72 h. Under cold storage conditions under microaerophilic conditions, there was an initial decline in *C. coli* at both 4°C and 0°C. After 6 h at 4°C, the level was reduced to approximately 3 log CFU/ml, and only decreased by another 1 log CFU/ml through 24 h. After 6 h at 0°C, the level of the organism decreased by approximately 5 log CFU/ml, but did not decrease over the following 18 h.

     Acid tolerance. Testing was conducted to determine if the selected rifampicin-resistant *Salmonella* and *Y. enterocolitica* and the respective parent organisms had similar sensitivity to lowered pH due to lactic acid. Both the parent and rifampicin-resistant *Salmonella* had consistent survival as pH declined from 7.0 to 4.5, but showed no survival at pH 4.0, 3.5, and 3.0. The parent and rifampicin resistant *Y. enterocolitica* showed similar growth from pH 7.0 to 5.5, but had both showed no growth at or below pH 5.0. Both of the rifampicin resistant strains showed similar sensitivity to lactic acid as compared to their parent counterparts. This supports that use of these selected strains

as surrogates for the parent strains of *Salmonella* and *Yersinia* for evaluation of

decontamination methods on variety meats.

     Inoculation trials. Inoculation methods and bacterial attachment were tested for

Salmonella, Y. enterocolitica, and *C. coli*. Initial inoculum levels were approximately 8

log CFU/ml, as shown in Table 3, for both *Salmonella* and *Y. enterocolitica*, and 7 log

CFU/g for *C. coli*. Two inoculation methods were compared, a dip (or immersion) and a

drip method. Two attachment times, 10 and 30 min, were tested using each inoculation

method. After the allotted attachment time, each sample was rinsed and plated

according to the methods for microbiological analysis. The results, as shown in Table 3,

indicate that the drip method with 30 min of attachment yielded the best attachment for

Y. enterocolitica. The dip method with 10 min attachment and the drip method with 30

min attachment resulted in similar attachments of *Salmonella* (6.3 and 6.1 log

CFU/sample, respectively). However, during testing, the drip method was determined to

be preferred over dipping due to the volume of inoculum required to dip the variety

meats and the ease of application of the drip method. *C. coli* was tested using the drip

method with 30 min attachment. Results of the inoculation trials determined that the

drip inoculation method was best suited for this application due to the ease of preparing,

inoculating, and cleanup of the inoculum. The drip method also yielded sufficient levels

of the organisms to show reductions by the treatments.

Immediate treatment effects

There were significant (P<0.05) replicate interactions (replicate x storage time and replicate x treatment) reported within treatments (data not shown). Replicate interactions (P<0.05) were also observed for reductions of all microorganisms (*Salmonella, Y. enterocolitica, C. coli,* APC, ECC, and TCC) at the freezing (FR) step and for all microorganisms except *Salmonella* at the chilling (CH) step. This shows that the treatments used in this study were inconsistent in their effectiveness on different days.

The least squares means of the initial microbial levels on variety meats are presented in Table 4. The initial inoculum levels of *Salmonella* and *Y. enterocolitica* following inoculation and attachment were very consistent (6.1-6.3 log CFU/sample and 5.0-5.1 log CFU/sample across variety meats, respectively). There were significant (P<0.05) main effects caused by replicate for initial levels of *C. coli*, APC, *E. coli,* and coliforms. This was likely due to variation in initial levels of naturally occurring microorganisms.

Water washing resulted in slightly larger reductions of microorganisms on inoculated variety meats than on non inoculated variety meats (Tables 5-10). This was most likely because the inoculated bacteria were less firmly attached to the surface of intestines than the surfaces of the other variety meats. For *Salmonella* and *Y. enterocolitica,* both spraying steps (WW and LA) resulted in the lowest reductions on intestines when compared to other variety meats, as reported in Tables 5 and 6. It is also likely that the mucosal layer of the intestine, which aids in absorption, secretion, and

digestion in the live animal, enhanced the attachment of bacteria and also prevented contact of the antimicrobial and the bacteria *(5, 42)*. While water washing alone resulted no reduction in levels of *Salmonella* on intestines, there were reductions of 0.1, 0.4, and 0.6 log CFU/sample on stomachs, hearts, and livers, respectively. When water washing was followed by a lactic acid spray, there were slightly greater reductions in levels of *Salmonella* (0.6 log CFU/sample) on livers, hearts, and stomachs, with the lowest reduction in levels of *Salmonella* on intestines (0.3 log CFU/sample). Results of samples analyzed after chilling alone showed mean reductions in levels of *Salmonella* of 0.1 log CFU/sample; however, when the water wash and lactic acid spray were applied before chilling, the reduction in levels was 0.6 log CFU/sample. Once all samples were frozen, there were significantly greater reductions (P<0.05) in levels of *Salmonella*, observed on stomachs than on other variety meats. The main effect of treatment was significant (P<0.05). Treatment 1 (WW+LA+FR) had higher reductions in levels of *Salmonella* when compared to other treatments (1.4 log CFU/sample). Samples from both Treatments 2 (FR) and 4 (CH + FR) had lower reductions in levels of *Salmonella* (0.6 and 0.5 log CFU/sample), and also had no spray treatments applied.

All treatment steps resulted in similar reductions in levels of both *Y. enterocolitica* and *Salmonella.* Reductions in levels of *Y. enterocolitica* (Table 6) after the water wash ranged from 0.2 to 0.8 log CFU/sample, and reductions in levels of *Y. enterocolitica* after the lactic acid spray ranged from 0.5 to 1.2 log CFU/sample. The main effect of variety meat was significant (P<0.05) for chilling and freezing. Significantly higher reductions (P<0.05) were observed on stomachs (0.7 log

CFU/sample) when compared to other variety meats, regardless of treatment. Spraying variety meats with water and lactic acid before chilling resulted in a 0.7 log CFU/sample reduction in levels of *Y. enterocolitica*, which is a significantly larger reduction (P<0.05) than achieved by chilling alone. Treatment 1 (WW + LA + FR) was most effective, resulting in a reduction of 1.6 log CFU/sample of *Y. enterocolitica*. Treatment 3 (WW + LA + CH + FR), which also incorporated lactic acid as a decontamination step, resulted in a reduction in levels of *Y. enterocolitica* by 1.2 log CFU/sample. Overall, the treatments without spraying steps had significantly lower reductions (P <0.05) in levels of *Y. enterocolitica* than those with spraying steps.

The effects of the treatment steps on *C. coli* are presented in Table 7. After both chilling and freezing, larger reductions in levels of *C. coli* were observed than for *Salmonella* and *Y. enterocolitica*. It is important to note that the root mean square error for the *C. coli* ranged from 0.81 to 1.30 log CFU/sample, which shows that there was a larger range in the data with this organism than with the previous organisms. The water wash step was very effective in reducing levels of *C. coli* on livers, with a mean reduction of 2.1 log CFU/sample, which was significantly higher than for the other 3 variety meats. However, the mean reduction for stomachs showed that there was an increase in levels of *C. coli*. This may be due to increased variation in the range of microbial counts, as indicated by the root mean square error. In addition, the mean reductions on hearts, stomachs, and intestines are not statistically different (P>0.05). Reductions after lactic acid spraying ranged from 0.7 to 1.2 log CFU/sample. The application of lactic acid before chilling resulted in a significantly (P<0.05) higher

reduction in levels of the organism (1.6 log CFU/sample) than chilling with no

decontamination treatment (1.1 log CFU/sample). Freezing caused a minimum mean

reduction in levels of *C. coli* of 2.1 log CFU/sample. Treatment 1 (WW + LA + FR)

resulted in significantly larger reductions than the other treatments (3.6 log

CFU/sample). Similar to treatment effects on *Salmonella* and *Y. enterocolitica,* the

treatments that did not include a decontamination step of water or lactic acid resulted in

significantly lower reductions (P<0.05) that the other treatments. The reductions in

levels of *C. coli* were 2.1 and 2.3 log CFU/sample for Treatment 4 (CH + FR) and 2

(FR), respectively, showing that reducing the temperature of the products by chilling and

freezing alone had relatively large reductions in levels of *C. coli*.

Because plants do not introduce pathogens into their processes for validation,

indicator organisms are used. In this study, the reductions in levels of APC, ECC, and

TCC were used to evaluate their use as indicator organisms in plants, and results are

presented in Tables 8, 9, and 10. Reductions in levels of APC, ECC, and TCC on non

inoculated variety meats were low for water wash, and some levels actually increased.

However, there were no significant differences among variety meats. Lactic acid

treatment reduced levels of APC, ECC, and TCC by 0.3-1.4 log CFU/sample. The

highest reductions were observed on hearts. Treatments that included decontamination

sprays (WW and LA) resulted in higher reductions after chilling and freezing. Growth

of APC, ECC, and TCC occurred during chilling without any decontamination treatment

before chilling. The mean growth for APC was 0.3 log CFU/sample, and the growth for

ECC and TCC was 0.2 log CFU/sample. While Treatment 1 (WW + LA + FR)was more

effective than all other treatments at reducing levels of pathogens tested, Treatment 1 (WW + LA + FR) and Treatment 3 (WW + LA + CH + FR) are not statistically different (P>0.05) in effectiveness at reducing APC, ECC, and TCC. Both treatments reduced APC by 1.3 log CFU/sample, and ECC and TCC by 1.3-1.5 log CFU/sample after freezing.

Overall, the treatments that use lactic acid as a decontamination treatment before chilling and freezing had the greatest reductions. Treatment 1 (WW + LA +FR) was the most effective treatment for reducing levels of pathogens, but did not differ (P>0.05) from Treatment 3 (WW + LA + CH + FR) for reducing levels of APC, ECC, and TCC on non inoculated products. Treatment 5 (WW + FR) was more effective than the treatments that did not include a water wash or lactic acid spray (Treatments 2 (FR) and 4 (CH + FR)) at reducing levels of pathogens, but was not different for reductions in APC, ECC, and TCC on non inoculated samples. These data support the hypothesis that use of water wash and a lactic acid spray before chilling variety meats is more effective in reducing levels of microorganisms.

Effects of treatments during storage

There were significant (P<0.05) replicate interactions (replicate x storage time and replicate x treatment) reported during shelf life (data not shown). Significant replicate interactions (P<0.05) were reported for reductions in all microorganisms (*Salmonella, Y. enterocolitica, C. coli,* APC, ECC, and TCC) on livers. Reductions in levels of *C. coli* on stomachs and hearts also showed replicate interactions. Significant

replicate interactions (P<0.05) were reported for reductions in both ECC and TCC on all

variety meats. Overall, this shows that the treatments used do not result in consistent

reductions.

As shown in Table 11, the main effects of storage time and treatment were

significant (P<0.05) for log reductions of *Salmonella* over the frozen shelf life. The two

way interactions of treatment by storage time are presented in Figure 2. For all variety

meats, reductions in levels of *Salmonella* at 0 storage time were significantly smaller

(P<0.05) than reductions after 2, 4, and 6 months of storage time. The largest decline in

levels of *Salmonella* occurred within the first 2 months of frozen storage, while smaller

reductions were observed after 4 months and 6 months, as displayed by the slopes of the

lines in Figure 2. After freezing overnight (0 storage time) reductions in levels of

Salmonella ranged from 0.6-1.3 log CFU/sample, while greater reductions in levels of

Salmonella (1.2-2.8 log CFU/sample) were observed after storage for 2 months. At the

end of frozen storage, there were significantly greater reductions (P<0.05) when

compared to 0 storage time for intestines, stomachs, livers, and hearts (2.6, 3.3, 1.8, and

1.6 log CFU/sample, respectively) across all treatments. Across all storage times, the

trend showed that the largest reductions in levels of *Salmonella* were on stomachs,

followed by intestines, livers, and hearts, respectively.

For the reductions in levels of *Salmonella* due to the main effect of treatment

within intestines, Treatments 1 (WW + LA + FR) and 3 (WW + LA + CH + FR) resulted

in the greatest reductions (2.5 and 2.3 log CFU/sample, respectively). Treatments 1 and

3 were not different (P>0.05) from each other, but Treatment 3 was also not significantly

different (P>0.05) from Treatment 5 (WW + FR) (2.2 log CFU/sample). This shows that

spraying intestines with either water wash or a combination of water wash and lactic

acid resulted in larger reductions in levels of *Salmonella* than chilling and freezing

alone. Treatments 2 (FR) and 4 (CH + FR) resulted in reductions in levels of *Salmonella*

of 1.9 and 1.8 log CFU/sample, respectively. Stomachs had the largest overall

reductions in levels of *Salmonella* as compared to the other variety meats. Treatment 1

(WW + LA + FR) resulted in significantly larger reductions (P<0.05) in levels of

Salmonella than the other treatments (3.2 log CFU/sample). Reductions in levels of

Salmonella on stomachs during shelf life for Treatments 2, 3, 4, and 5 ranged from 2.2-

2.8 log CFU/sample.

For the main effect of treatment within livers and hearts, reductions in levels of

Salmonella following Treatments 1 (WW + LA + FR) and 3 (WW + LA + CH + FR)

were greater than the other treatments, though not significantly different from each other

(P>0.05) during shelf life. Least squares means of reductions in levels of the organism

on livers and hearts following Treatments 1 and 3 were 1.8 log CFU/sample. Treatment

5 (WW + FR) was less effective than the treatments that included a lactic acid spray

(Treatments 1 (WW + LA + FR) and 3(WW + LA + CH + FR)), though more effective

than treatments that did not include any spray treatment (Treatments 2 (FR) and 4 (CH +

FR). Reductions in levels of *Salmonella* following Treatment 5 (WW + FR) were 1.5

and 1.2 log CFU/sample for livers and hearts, respectively. Within each variety meat,

Treatments 2 (FR) and 4 (CH + FR) resulted in lower reductions in levels of *Salmonella*

(0.7-1.0 log CFU/sample) than treatments that included a water wash or lactic acid spray before storage.

The trends for reductions in levels of both *Salmonella* and *Y. enterocolitica* were similar. Results for main effects for *Y. enterocolitica* are shown in Table 12 and the two way interactions of treatment by storage time are shown in Figure 3. The intestines were the only variety meat that had a significant two way interaction of treatment by storage time (P=0.006) for reductions in levels of *Y. enterocolitica.* For intestines, livers, and hearts, the reductions in levels of *Y. enterocolitica* for the main effect of storage time were significantly lower (P<0.05) after 0 storage time (0.6-1.0 log CFU/sample) than 2, 4, or 6 months of storage. For stomachs, there was no difference (P>0.05) in reductions in levels of *Y. enterocolitica* between 0 storage time and 2 months storage time (1.2 and 1.9 log CFU/sample, respectively). Least squares means of reductions in levels of *Y. enterocolitica* at the end of shelf life (6 months) ranged from 1.7-2.5 log CFU/sample across all treatments.

Across all variety meats and storage times, Treatment 1 (WW + LA + FR) resulted in the greatest reductions in levels of *Y. enterocolitica,* although Treatments 1 (WW + LA + FR) and 3 (WW + LA + CH + FR) were not different (P>0.05) within livers and hearts. Reductions in levels of *Y. enterocolitica* due to Treatment 1 (WW + LA + FR) ranged from 2.0-2.8 log CFU/sample across variety meats. The trend showed that Treatments 1 (WW + LA + FR) and 3 (WW + LA + CH + FR) were most effective, followed by Treatment 5 (WW + FR), and Treatments 2 (FR) and 4 (CH + FR) were least effective in reducing levels of *Y. enterocolitica.* However, the least squares means

for all treatments over the storage time were equal to or greater than 1.0 log

CFU/sample.

Results of reductions in levels of *C. coli* during shelf life are shown in Table 13,

and two way interactions of treatment by storage time are shown in Figure 4. There

were significant two way interactions (P<0.05) of treatment by storage time for both

stomachs and hearts. Across all variety meats and treatments, 0 storage time resulted in

the lowest (P<0.05) reductions in levels of *C. coli* when compared to the other storage

times. The initial reduction in levels of the organism due to overnight freezing (0

storage time) ranged from 2.2-3.3 log CFU/sample. Levels of *C. coli* continued to

decline over frozen storage, and the reductions after 6 months of storage ranged from

3.8-5.3 log CFU/sample. Across all storage times, the largest reductions in levels of *C. coli* were seen on intestines, followed by stomachs, livers, and hearts, respectively.

Across all storage times, there was less variation among the treatments in the

reductions in levels of *C. coli* than in levels of *Salmonella* or *Y. enterocolitica*. This was

likely due to the greater susceptibility of *C. coli* to drying that occurs during freezing

(Oosterom, 1983). For all variety meats, the reductions in levels of *C. coli* due to the

main effect of treatment ranged from 2.9-5.0 log CFU/sample. However, the rank of the

treatments in order of effectiveness at reducing levels of *C. coli* varied across variety

meats. Frozen storage most likely had the largest effect on the levels of the organism,

regardless of the treatment applied before storage.

Results of reductions in levels of APC during frozen shelf life are shown in Table

14 and the two way interactions of treatment by storage time are shown in Figure 5.

Across all treatments, the lowest reduction in levels of APC (P<0.05) was after 0 storage time as compared to 2, 4, or 6 months storage time. Reductions in levels of APC after overnight freezing (0 storage time) ranged from 0.5-1.3 log CFU/sample, while the range after 2 months of frozen storage was 1.1-2.4 log CFU/sample. At the end of shelf life (6 months) reductions in levels of APC ranged from 1.3-2.9 log CFU/sample.

For the main effect of treatment on intestines and liver, Treatment 1 (WW + LA + FR) resulted in significantly larger reductions (P<0.05) than all other treatments. For hearts, Treatment 1 (WW + LA + FR) and 3 (WW + LA + CH + FR) resulted in the largest reductions (2.3 and 2.7 log CFU/sample, respectively). The mean for Treatment 1 (WW + LA + FR) effects on stomachs showed the largest reduction, but was not different than Treatments 2 (FR) and 3 (WW + LA + CH + FR). Except for stomachs, reductions in levels of APC due to Treatments 2 (FR) and 4 (CH + FR) were significantly lower (P<0.05) than the other treatments. On intestines, hearts, and livers, Treatment 5 (WW + FR) was less effective than treatments that included lactic acid, but was more effective than the treatments that did not have a water wash or lactic acid spray.

Results of reductions in levels of ECC were similar to TCC and are shown in Tables 15 and 16. Two way interactions of treatment by storage time are shown in Figures 6 and 7. Significant interactions (P<0.05) were observed for reductions in levels of ECC on intestines and hearts and for reductions in levels of TCC on stomachs. For the main effect of storage time, the lowest reductions (P<0.05) in levels of ECC and TCC were observed after 0 storage time for all variety meats (0.6-1.2 and 0.7-1.2 log

CFU/sample, respectively). At the end of shelf life storage (6 months), reductions in

levels of ECC and TCC were 2.1-3.4 and 2.0-3.5 log CFU/sample, respectively.

Reductions in levels of these microorganisms after 6 months of storage were

significantly greater (P<0.05) than reductions at the beginning of shelf life for all variety

meats.

For the main effect of treatment on reductions of levels of ECC and TCC, the

largest reductions (P<0.05) were reported for Treatment 1 (WW + LA + FR) on

intestines, stomachs, and livers. Reductions of levels of both ECC and TCC for

Treatment 1 (WW + LA + FR) were 2.2-3.5 log CFU/sample. Treatment 3 (WW + LA

+ CH + FR) resulted in the largest mean reduction in levels of ECC and TCC on hearts,

although it was not different (P>0.05) from Treatment 1 (WW + LA + FR) for reducing

levels of ECC. Reductions in levels of ECC on hearts for Treatment 1 (WW + LA + FR)

and 3 (WW + LA + CH + FR) were 2.9 and 3.2 log CFU/sample, respectively. Levels of

TCC were reduced by 2.9 and 3.3 log CFU/sample for Treatments 1 (WW + LA + FR)

and 3 (WW + LA + CH + FR), respectively. Treatment 5 (WW + FR) was more

effective than Treatments 2 (FR) and 4 (CH + FR), but less effective than the treatments

that included a lactic acid spray (Treatments 1 and 3). Across all treatments, least

squares mean reductions of levels of ECC and TCC were equal to or greater than 1.0 log

CFU/sample.

The least squares means of the two way interactions between treatment and

process point for pH are presented in Figure 8. Least squares means were separated

using pdiff (SAS) (data not shown). Across all variety meats, the pH was significantly

higher after the water wash (Treatment 5 x Spray) (pH 6.0-6.8) when compared to the

initial pH, which was expected due to the neutrality of water. Immediately after

spraying the variety meats with a combination of water wash followed by lactic acid, the

pH was significantly (P<0.05) lower (pH 3.8-4.0) than both initial pH and pH of samples

only sprayed with water. However, mean pH for all treatments were not different

(P>0.05) for stomachs and livers after 2 months and 4 months, respectively. The pH for

Treatments 1 (WW + LA + FR) and 3 (WW + LA + CH + FR) was significantly lower

(P<0.05) than the pH for Treatment 5 (WW + FR) throughout shelf life for intestines and

hearts. The differences in the means after 6 months of frozen storage across treatments

were within 0.8 and 0.3 pH units for intestines and hearts, respectively. It is likely that

the pH effect due to lactic acid was buffered over storage time.

DISCUSSION

This study showed that a 2% lactic acid spray resulted in significant reductions in levels of microorganisms on variety meat surfaces. Lactic acid has previously been shown to effectively reduce microbial levels on beef, buffalo, and pork variety meats *(14, 39, 45, 64, 66)*. Patterson and Gibbs *(39)* reported a 2 log CFU/cm^2 reduction in levels of APC on beef livers dipped in 1% lactic acid (7$^\mathbf{o}$C) for 15 min with agitation. Woolthuis et al. *(64)* reported that after pork livers were immersed in a 0.2% lactic acid solution for 5 min, were vacuum packaged, and stored for 1 day (3$^\mathbf{o}$C), levels of APCs were 2.2 log CFU/cm^2 lower than controls. After storage for 5 days (3$^\mathbf{o}$C), levels of APCs were approximately 3 log CFU/cm^2 lower when compared to controls *(64)*.

Selvan et al. *(45)* dipped buffalo variety meats in solutions of 2% lactic acid (ambient temperature, 10 s) and reported reductions in levels of APC and TCC of approximately 1 and 1.2 log CFU/g, respectively, when compared to controls. Delmore et al. *(14)* treated beef variety meats with a 2% lactic acid spray (10 s) and reported reductions in levels of APC, ECC, and TCC (0.4-1.7, 0-1.8, -0.1-2.5 log CFU/g, respectively). Zerby et al. *(66)* observed reductions of levels of APC, ECC, and TCC on pork variety meats after spraying with 2% lactic acid (10 s, 48-50$^\mathbf{o}$C). The reductions of the levels of APC ranged from 1.1-4.1 and 1.6-4.5 log CFU/g, respectively *(66)*.

Zerby et al. *(66)* observed reductions in levels of *Salmonella* on inoculated pork variety meats after spraying with 2% lactic acid (10 s, 48-50$^\mathbf{o}$C). Reductions in levels of *Salmonella* of approximately 2 log CFU/g were reported on livers, stomachs, and hearts,

while reductions of approximately 3 log CFU/g were observed on chitterlings. In the same study, chitterlings were inoculated with *Y. enterocolitica* and immersed in 2% lactic acid (10 s, 48-50°C), which reduced levels of *Y. enterocolitica* below detectable levels *(66)*.

However, the overall effectiveness of lactic acid varied based on the variety meat surfaces. Water washing has been shown to be effective at reducing microbial levels of carcasses *(4)*. Hardin et al. *(23)* showed that a water wash (35°C) followed by a 2% lactic acid wash (55°C) significantly reduced *E. coli* O157:H7 and *Salmonella* on beef surfaces. However, the treatment was less effective on beef inside rounds because of the surface variation where bacteria could become embedded or attached and not be contacted by the sprays *(23)*. A similar situation was reported by Ellebracht et al. *(15)* when a 3 s hot water treatment was used to treat beef trimmings. Ellebracht et al. *(15)* noted that not all of the fat and lean surfaces were evenly exposed to the treatment because of overlap of the trim pieces.

A similar situation could have occurred in this study because of the vast differences in variety meat surfaces. While the liver has a very smooth, regular surface, the intestine, heart, and stomach have more irregular, convoluted surfaces areas. These differences in surface types may have prevented even exposure of the surface and associated microorganisms to the water wash and lactic acid spray. In addition, the variation in variety meat surfaces, particularly the slimy mucosal layer of the intestine, could have similar compounding effects on all treatments. Woolthuis et al. *(64)* reported that different sites of the liver surface were contaminated with different levels of blood

or digestive contents, which may have contributed to variation within decontamination treatments. It is possible that a longer dwell time, increased temperature, increased volume, or a dip application method could increase effectiveness of these treatments.

Patterson and Gibbs *(39)* reported reductions in levels of APC by approximately 2 log CFU/cm^2 on chilled hearts and livers after immersion in 1% lactic acid for 15 min. Snijders et al. *(49)* reported that the immediate effect of lactic acid on levels of APCs on both carcasses and variety meats was approximately 1.5 log CFU/g. A delayed bactericidal effect was observed during storage and was suspected to be due to the extension of the lag phase of injured organisms after lactic acid treatment *(49)*. While it may be more effective for plants to implement one method for treatment of all variety meats, one method may not be appropriate for all types of variety meats. Effects on quality would also need to be taken into consideration before determining treatments. Delmore et al. *(14)* reported that immersion of livers in 2% acetic acid or 2% lactic acid resulted in a lighter color as compared to controls. Woolthuis et al. *(64)* reported no effect on color when livers were immersed in 0.2% lactic acid for 5 min. However, Patterson and Gibbs *(39)* reported that livers immersed in hot water (90°C) for 1 minute exhibited a cooked appearance. This suggests that concentration of the organic acid and temperature control of decontamination treatments is critical particularly for livers because of the denaturation of myoglobin and the impact on color.

Immediately after spraying with lactic acid, lower pH values were reported on all variety meats. Woolthuis *(64)* reported significantly lower (P<0.05) pH values on livers treated with 0.2% lactic acid for 5 min after 1 day of storage (2-4°). However, pH

values after 5 days of storage (2-4°) were not different from controls *(64).* The pH of samples treated with lactic acid was lower immediately after spraying than initial pH, which is similar to results reported by Woolthuis et al. *(64).* Application of 1.25% lactic acid to hot calf carcasses reduced pH by over 3 units initially, but the pH returned to the original level after 72 h *(65).* After freezing and throughout shelf life, pH differences across treatments were quite small in this study. In this study, pH values after freezing and throughout shelf life storage were not different between treatments except for intestines.

The reduction in levels of microorganisms due to freezing depends on the medium, as some media protect microorganisms while others enhance damage by freezing *(30).* Blast chilling was not shown to have an effect on the prevalence of *Y. enterocolitica* or the levels of APCs on pork carcasses, but did significantly reduce the prevalence of *Campylobacter* spp. and the levels of coliforms and *E. coli (34).* Stern and Kotula *(52)* reported that levels of *Campylobacter* were reduced by 3 log CFU/g after 3 days of frozen storage (-15°C). Gill and Harrison *(19)* reported variation in levels of *E. coli* on variety meats based not only on the length of the chilling curve, but also type of variety meat and batch of variety meat.

Epling et al. *(16)* reported that a 2% lactic acid spray on pork carcasses reduced levels of both *Salmonella* and *Campylobacter* both 5 min after spraying and after 20 h of chilling at 4°C. The prevalence of *Campylobacter* spp. was reduced to a greater extent on carcasses that were chilled using conventional chilling as compared to a spray chilling system *(16).* This is likely due to the increased drying of the surface during

conventional chilling. This was also observed when blast chilling was compared to conventional chilling for reducing *Campylobacter* spp. *(12)*.

The results of this research showed that washing variety meats with water (25°) for 10 s did not reduce levels of APC, ECC, or TCC. In fact, half of the least squares means of the reductions in levels of APC, ECC, and TCC after the WW step actually indicate that growth occurred. Lactic acid treatment immediately reduced all microorganisms (*Salmonella, Y. enterocolitica, C. coli,* APC, ECC, and TCC) by 0.3-1.4 log CFU/sample, similar to previous reports *(14, 66)*.

In the present study, growth of APC, ECC, and TCC was reported on non inoculated samples that were not sprayed with water or lactic acid before chilling (4°C) overnight. Hanna et al. *(22)* reported an increase in APC of pork livers, kidneys, and hearts over 5 days of aerobic storage at 2°C. Zerby et al. *(66)* treated pork hearts with lactic acid (2%, 50°C, 10 s immersion) and stored treated and untreated controls for 63 days (vacuum packaged, 3°C). Lower levels of APC and TCC throughout the shelf life were reported for treated samples as compared to untreated controls. Zerby et al. *(66)* reported that while there was a minimum 2.5 log CFU/g reduction in levels of APC after treating pork variety meats with 2% lactic acid (50°C, 10 s), brine chilling (-12°C, 45 min), and freezing (0°C for 12 h, followed by blast freezing at -12°C for 6 h), samples that . Zerby et al. *(66)* concluded that a multiple hurdle approach including a decontamination treatment such as lactic acid should be combined with GMPs for initial chilling of variety meats to reduce microbial contamination.

In general, during the shelf life storage time for this study (0°C, 6 months), the treatments that included a lactic acid spray resulted in the greatest reductions of all microorganisms (*Salmonella, Y. enterocolitica, C. coli,* APC, ECC, and TCC) regardless of whether the samples were chilled before they were frozen (≥ 1.7 log CFU/sample). The treatment that included only a water wash and freezing was intermediate (≥ 1.0 log CFU/sample) between those treatments that included lactic acid and those treatments that had no water wash or lactic acid (≥ 0.7 log CFU/sample). For the treatments without the application of lactic acid or water wash, there was no trend that indicated whether chilling before freezing affected reductions in levels of any of the microorganisms.

CONCLUSIONS

In this study, it is important to recognize that there were significant effects (P<0.05) due to replication and replicate interactions, suggesting that the effectiveness of the treatments to reduce microbial levels on variety meats varied each sample day. It is reasonable that variation in background levels of microbial contamination each day likely affect the ability of treatments to reduce levels of microorganisms. There were also significant interactions between treatment and storage time, suggesting that treatment and storage do not always produce an additive effect when reducing levels of microorganisms.

Overall, the use of 2% lactic acid as a decontamination intervention in addition to good GMPs (employee hygiene, sanitation, and rapid chilling) during processing of pork variety meats causes significant reductions in levels of *Salmonella, Y. enterocolitica,* and *C. coli*, as well as indicator organisms (APC, ECC, and TCC). However, reductions of equal to or greater than 1 log CFU/sample of the pathogens were observed on variety meats treated with only a water wash and subsequently frozen. Trends in levels of APC, ECC, and TCC are similar to trends in levels of pathogens and can thus be used as surrogates for pathogens to monitor and validate processes in plants.

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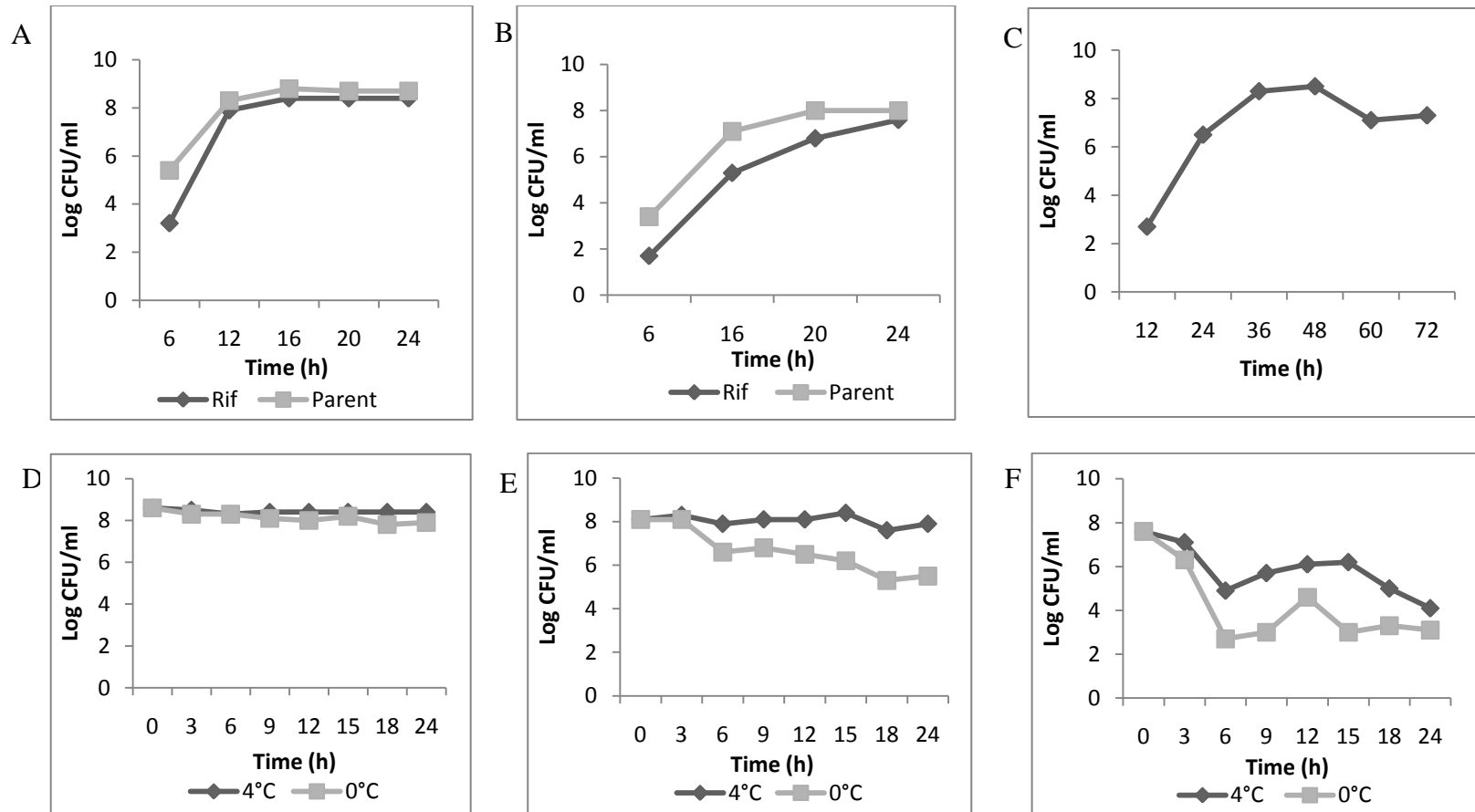
APPENDIX A

FIGURES AND TABLES

Figure 1. *Growth of (A) parent and rifampicin-resistant* Salmonella *at 35°C, (B) parent and rifampicin-resistant* Yersinia enterocolitica *at 35°C, (C)* Campylobacter coli *at 42°C, and survival of (D) rifampicin-resistant* Salmonella *at 4°C and 0°C, (E) rifampicin-resistant* Yersinia enterocolitica *at 4°C and 0°C, (F)* Campylobacter coli *at 4°C and 0°C.*
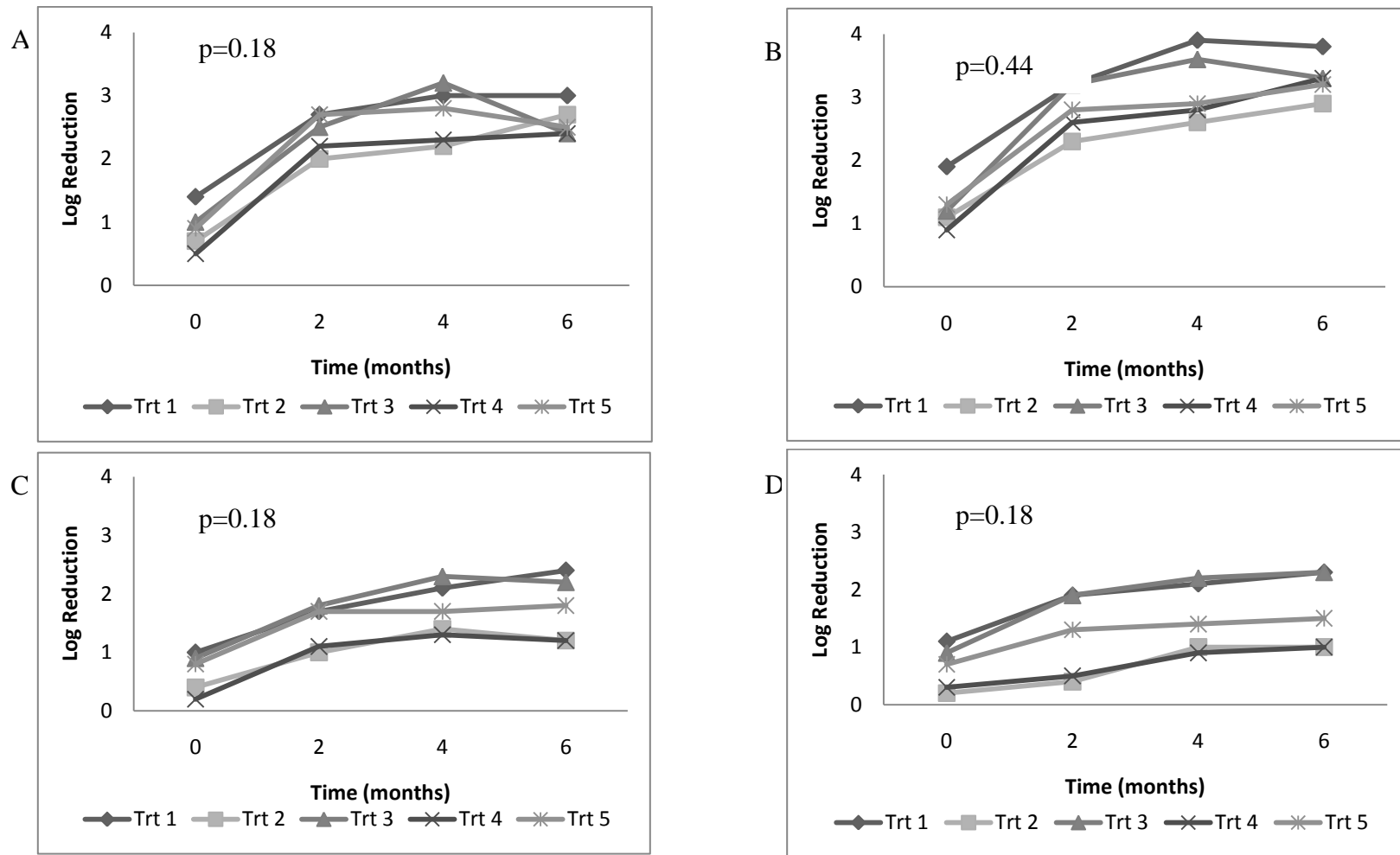
Figure 2. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of* Salmonella *on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*
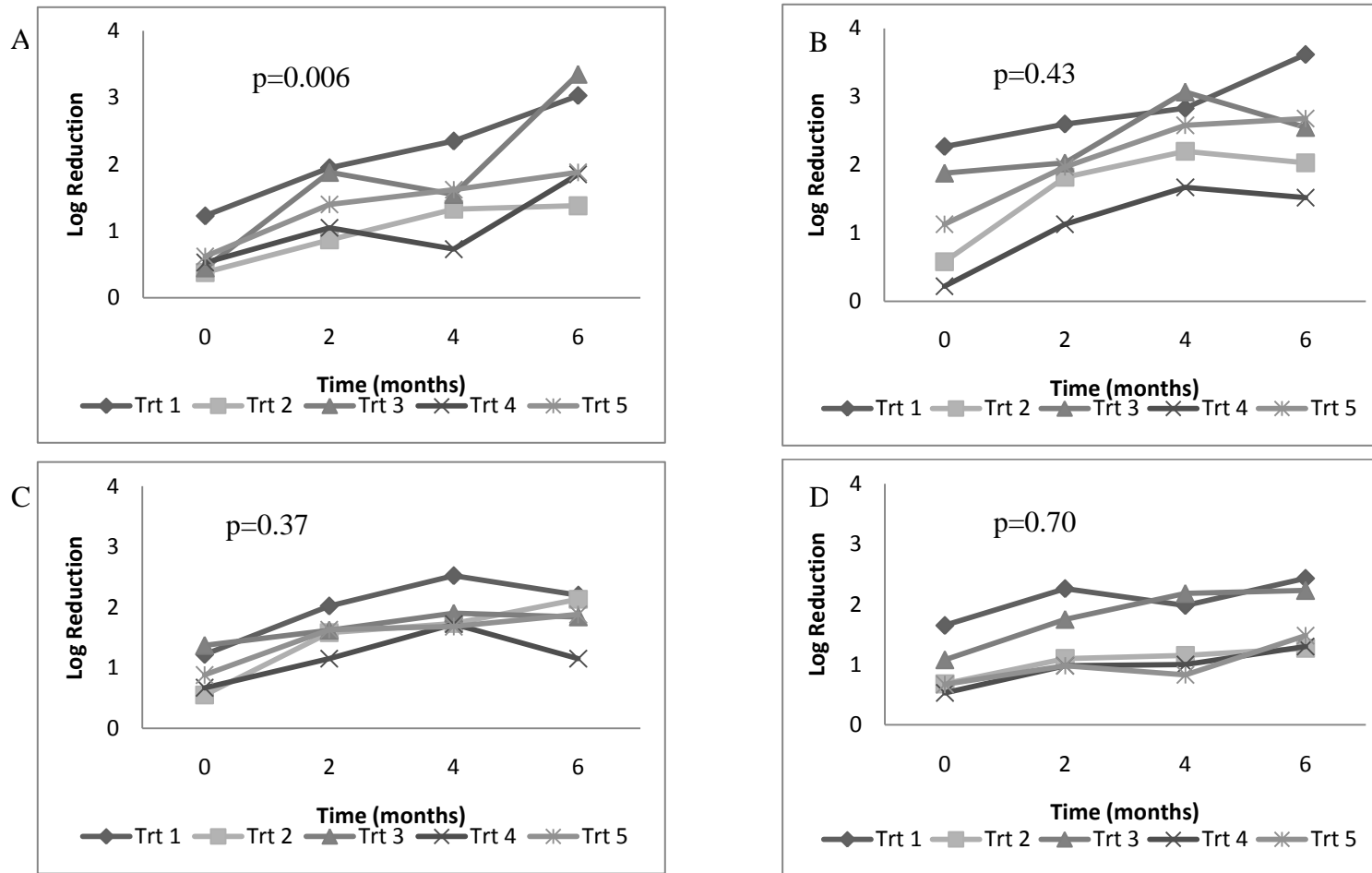
Figure 3. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of* Yersinia enterocolitica *on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*
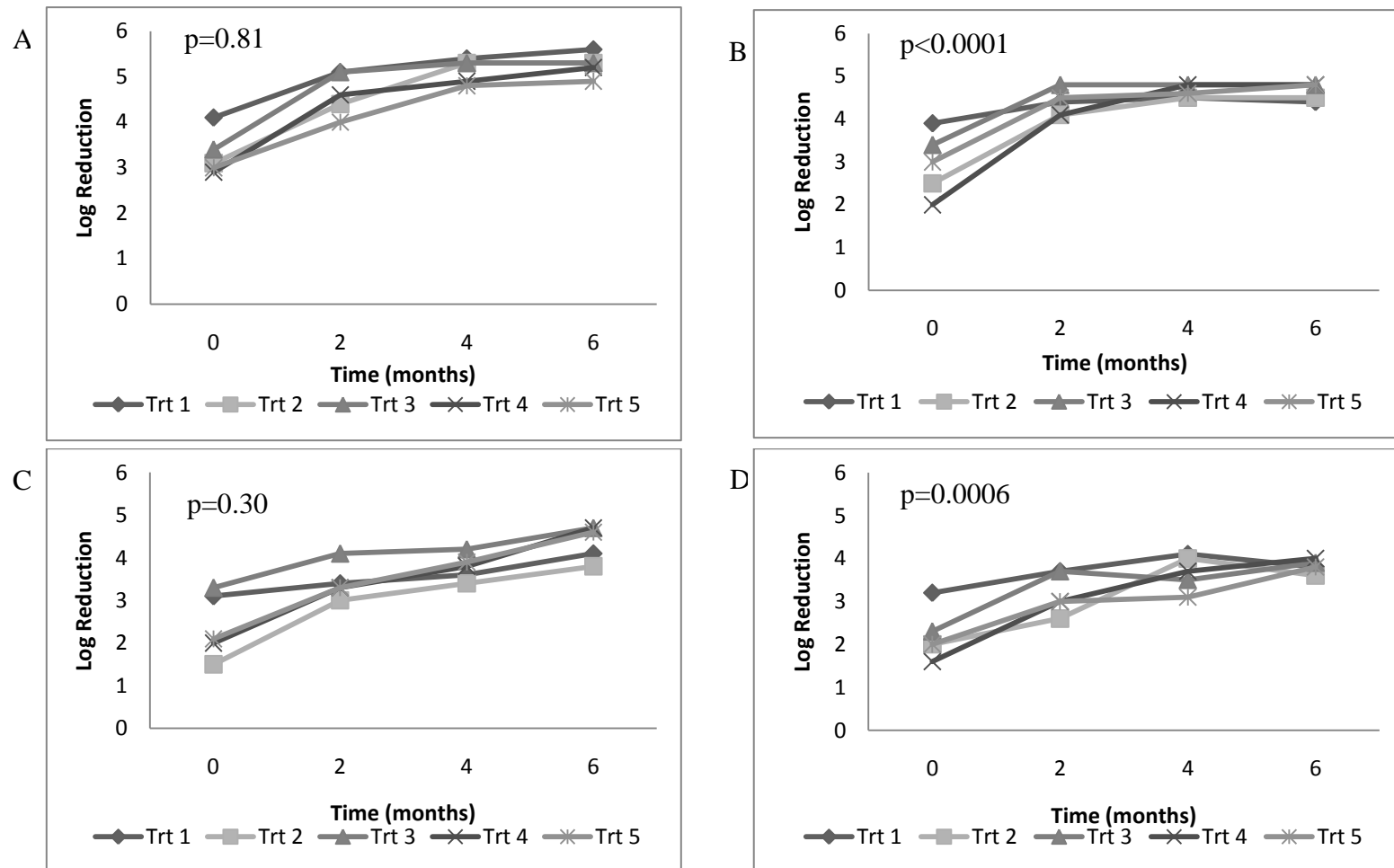
Figure 4. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of* Campylobacter coli *on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*
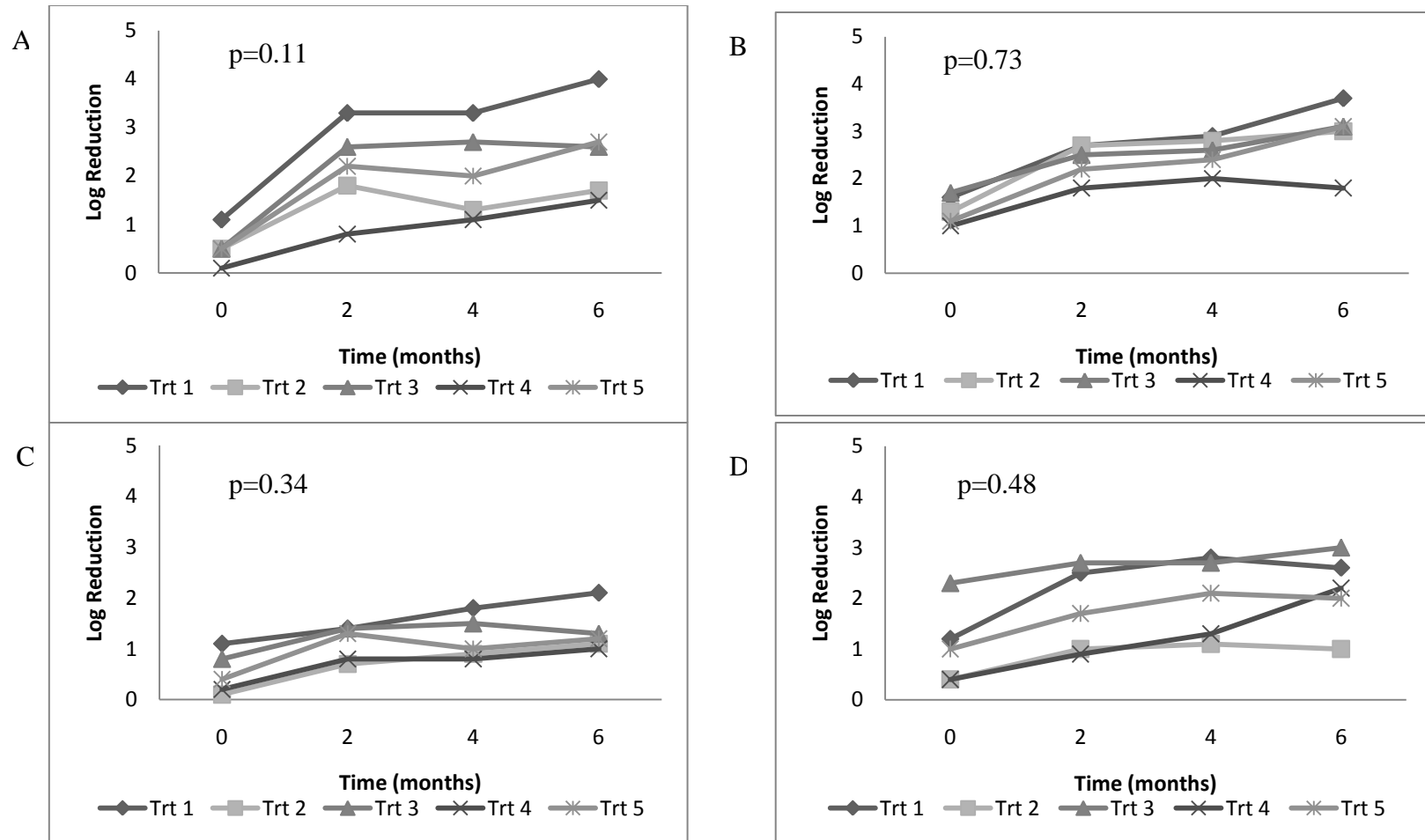
Figure 5. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of aerobic plate counts on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*

Figure 6. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of* Escherichia coli *on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*
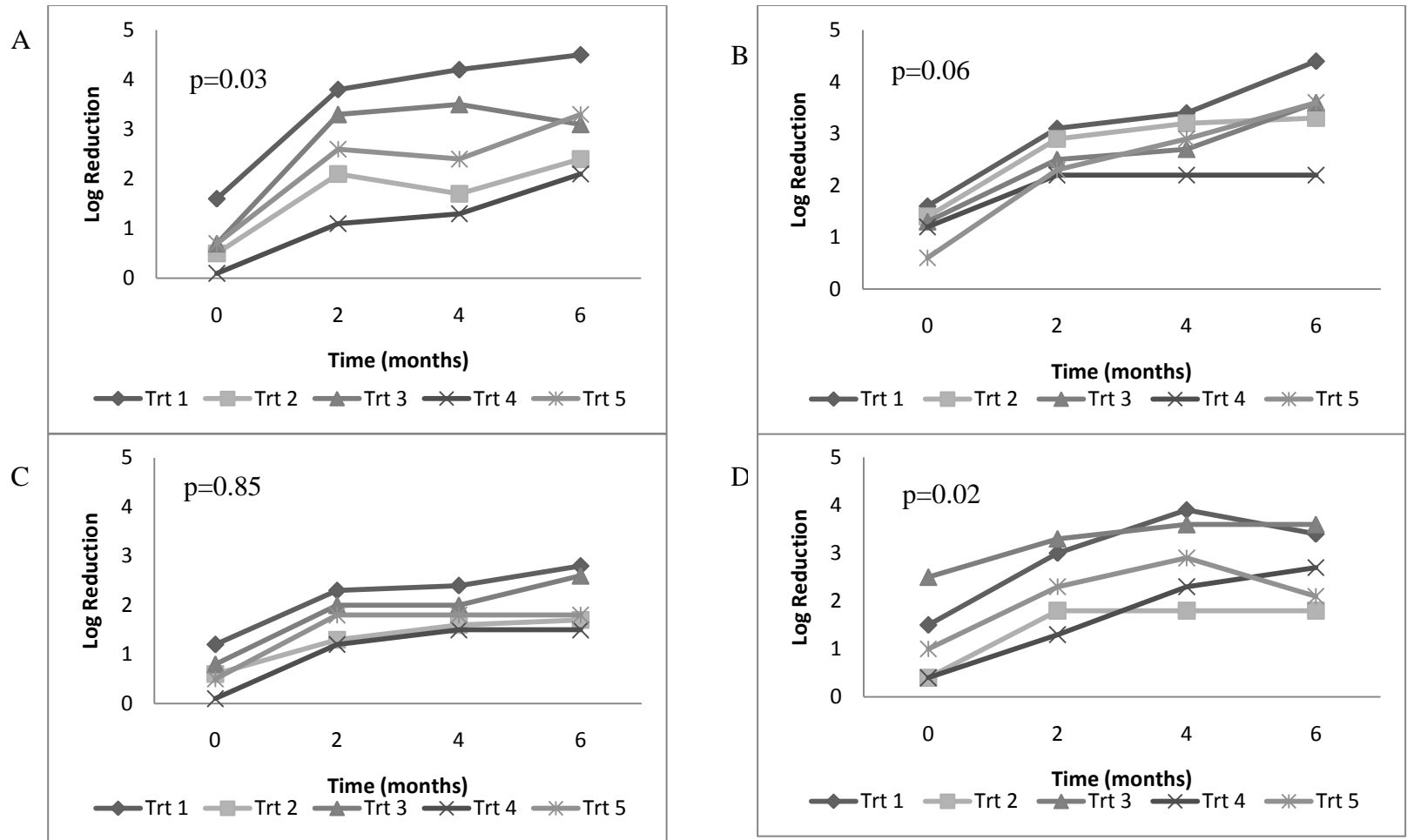
Figure 7. *Least squares means of the interaction between treatment and storage time of log reductions (log CFU/sample) of coliforms on (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 2: freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 4: chill + freeze; Treatment 5: water wash + freeze.*
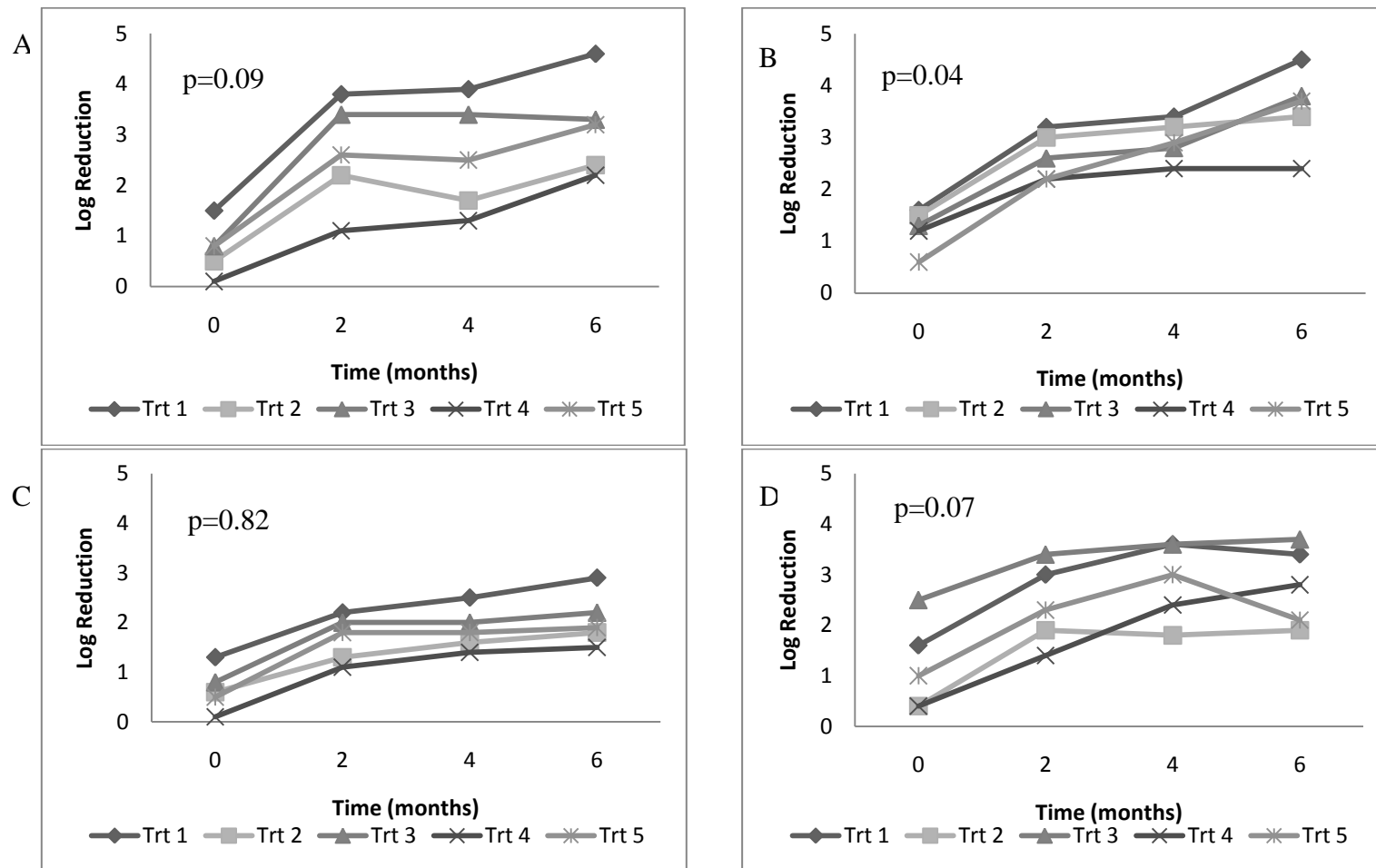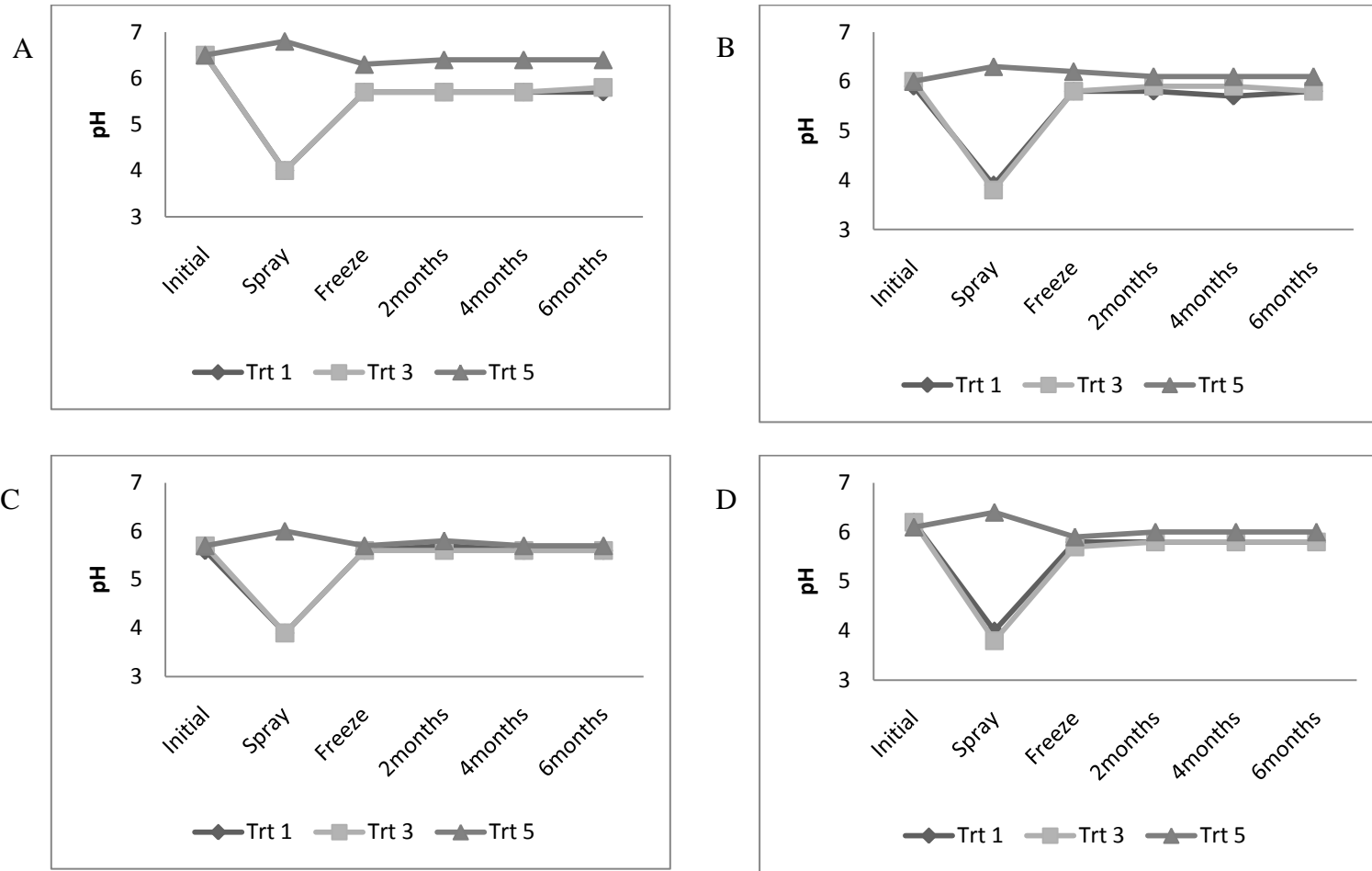
Figure 8. *Least squares means of the interaction between treatment and process point of pH of (A) intestine (B) stomach (C) liver (D) heart. Treatment 1: water wash + lactic acid + freeze; Treatment 3: water wash + lactic acid + chill + freeze; Treatment 5: water wash + freeze.*

Table 1. *Growth of rifampicin-resistant* Salmonella *and rifampicin-resistant* Yersinia enterocolitica *(log CFU/ml) on media with and without rifampicin.*

Media[a]	*Salmonella*	*Y. enterocolitica*
Without Rifampicin	8.4	8.3
With 100 µg/ml Rifampicin	8.5	8.2

[a] TSA and rif-TSA were used for *Salmonella* and Yersinia Selective Agar and rif-Yersinia Selective Agar were used for *Y. enterocolitica.*

Table 2. *Acid tolerance of parent and rifampicin-resistant* Salmonella *and* Yersinia enterocolitica *grown on TSA at varying pH levels (log CFU/ml).*

TSA pH	Parent *Salmonella*	Rif-*Salmonella*	Parent *Y. enterocolitica*	Rif-*Y. enterocolitica*
7.0	46	47	32	35
6.5	77	47	26	33
6.0	71	65	24	39
5.5	77	57	37	34
5.0	22	17	<1	<1
4.5	44	49	<1	<1
4.0	<1	<1	<1	<1
3.5	<1	<1	<1	<1
3.0	<1	<1	<1	<1

[a] Initial inoculum level was approximately 2.0 log CFU/ml.

Table 3. *Bacterial counts (log CFU/sample) after inoculation and attachment on pork livers.*

| | Log CFU/sample | | |
	Salmonella	*Yersinia enterocolitica*	*Campylobacter coli*
Inoculum[a]	8.4	8.1	6.9
Inoculuation Method[b]			
Dip +10 minute	6.3	5.1	-
Dip +30 minute	5.4	5.1	-
Drip +10 minute	5.7	5.4	-
Drip +30 minute	6.1	6.6	4.3

[a] 18 h culture of *Salmonella* and *Y. enterocolitica* and 48 h culture of *C. coli.*
[b] Inoculation method and time allowed for attachment before microbiological analysis.

Table 4. *Least squares means of initial microbial levels before application of treatments.*

| VarietyMeat | Log CFU/sample[a] | | | | | |
	Salmonella	*Yersinia enterocolitica*	*Campylobacter coli*	Aerobic plate counts	*Escherichia coli*	Coliforms
Intestine	6.3 A	5.1	5.3 A	6.6 B	6.2 B	6.4 B
Stomach	6.3 A	5.0	4.7 B	7.1 A	6.6 A	6.8 A
Liver	6.1 B	5.0	4.6 B	6.0 C	5.6 C	5.8 C
Heart	6.2 B	5.0	4.2 C	5.6 D	5.0 D	5.1 D

[a]Means within each column bearing a common letter are not significantly different ($P > 0.05$).

Table 5. *Least squares means of log reductions of* Salmonella *after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.0 B	0.3 B	0.3	0.9 B
Stomach	0.1 AB	0.6 A	0.4	1.3 A
Liver	0.6 A	0.6 A	0.3	0.7 C
Heart	0.4 A	0.6 A	0.3	0.6 C
Treatment				
1 WW+LA+FR	-	-	-	1.4 A
2 FR	-	-	-	0.6 C
3 WW+LA+CH+FR	-	-	0.6 A	1.0 B
4 CH+FR	-	-	0.1 B	0.5 C
5 WW+FR	-	-	-	1.0 B

[a]Initial inoculum level was 6.1-6.3 log CFU/sample.

[b]Sampled after water wash.

[c]Sampled after water wash followed by lactic acid spray.

[d] Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.

[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 6. *Least squares means of log reductions of* Yersinia enterocolitica *after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.2 B	0.5 B	0.2 B	0.6 C
Stomach	0.8 A	1.2 A	0.7 A	1.2 A
Liver	0.5 AB	0.8 AB	0.2 B	0.9 B
Heart	0.4 AB	0.8 AB	0.5 B	0.9 B
Treatment				
1 WW+LA+FR	-	-	-	1.6 A
2 FR	-	-	-	0.6 D
3 WW+LA+CH+FR	-	-	0.7 A	1.2 B
4 CH+FR	-	-	0.1 B	0.5 D
5 WW+FR	-	-	-	0.8 C

[a]Initial inoculum level was 5.0-5.1 log CFU/sample.
[b]Sampled after water wash.
[c]Sampled after water wash followed by lactic acid spray.
[d]Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.
[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 7. *Least squares means of log reductions of* Campylobacter coli *after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.3 B	0.8	1.1 B	2.2 B
Stomach	-0.3 B	1.2	1.8 A	3.2 A
Liver	2.1 A	1.1	0.7 B	2.5 B
Heart	0.2 B	0.7	1.8 A	3.0 A
Treatment				
1 WW+LA+FR	-	-	-	3.6 A
2 FR	-	-	-	2.3 C
3 WW+LA+CH+FR	-	-	1.6 A	3.1 B
4 CH+FR	-	-	1.1 B	2.1 C
5 WW+FR	-	-	-	2.6 B

[a]Initial inoculum level was 4.2-5.3 log CFU/sample.

[b]Sampled after water wash.

[c]Sampled after water wash followed by lactic acid spray.

[d]Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.

[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 8. *Least squares means of log reductions of aerobic plate counts (APCs) after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.0	0.3	-0.1	0.6 B
Stomach	-0.3	0.6	-0.1	1.3 A
Liver	0.0	0.3	0.0	0.5 B
Heart	0.1	0.7	0.4	1.1 A
Treatment				
1 WW+LA+FR	-	-	-	1.3 A
2 FR	-	-	-	0.6 BC
3 WW+LA+CH+FR	-	-	0.4 A	1.3 A
4 CH+FR	-	-	-0.3 B	0.4 C
5 WW+FR	-	-	-	0.8 B

[a]Initial inoculum level was 5.6-7.1 log CFU/sample.
[b]Sampled after water wash.
[c]Sampled after water wash followed by lactic acid spray.
[d]Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.
[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 9. *Least squares means of log reductions of* Escherichia coli *after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.2	0.4 B	0.1	0.7 B
Stomach	-0.1	0.5 B	0.0	1.2 A
Liver	-0.1	0.4 B	0.0	0.6 B
Heart	-0.1	1.4 A	0.5	1.2 A
Treatment				
1 WW+LA+FR	-	-	-	1.4 A
2 FR	-	-	-	0.7 B
3 WW+LA+CH+FR	-	-	0.5 A	1.3 A
4 CH+FR	-	-	-0.2 B	0.5 B
5 WW+FR	-	-	-	0.7 B

[a]Initial inoculum level was 5.0-6.6 log CFU/sample.

[b]Sampled after water wash.

[c]Sampled after water wash followed by lactic acid spray.

[d]Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.

[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 10. *Least squares means of log reductions of coliforms after steps within treatments (log CFU/sample)[a].*

	WW[b]	LA[c]	CH[d]	FR[e]
Variety Meat				
Intestine	0.2	0.3 B	0.1	0.8 B
Stomach	-0.1	0.6 B	0.0	1.2 A
Liver	0.1	0.3 B	0.0	0.7 B
Heart	-0.1	1.4 A	0.5	1.2 A
Treatment				
1 WW+LA+FR	-	-	-	1.5 A
2 FR	-	-	-	0.8 B
3 WW+LA+CH+FR	-	-	0.5 A	1.4 A
4 CH+FR	-	-	-0.2 B	0.5 C
5 WW+FR	-	-	-	0.7 B

[a]Initial inoculum level was 5.1-6.8 log CFU/sample.

[b]Sampled after water wash.

[c]Sampled after water wash followed by lactic acid spray.

[d]Sampled after internal temperature of samples reached 4°C with previous treatment components according to Treatment.

[e]Sampled after internal temperature of samples reached 0°C with previous treatment components according to Treatment.

Table 11. *Least squares means of log reductions of* Salmonella *(log CFU/sample) over 6 months of frozen storage*.

	Reductions (before-treatment minus after-treatment means) of *Salmonella* (log CFU/sample)[a]			
	Intestine	Stomach	Liver[b]	Heart
Storage time[c]				
0	0.9 C	1.3 C	0.7 C	0.6 C
2 months	2.4 B	2.8 B	1.4 B	1.2 B
4 months	2.7 A	3.2 A	1.7 A	1.5 A
6 months	2.6 AB	3.3 A	1.8 A	1.6 A
Treatment[d]				
1 WW+LA+FR	2.5 A	3.2 A	1.8 A	1.8 A
2 FR	1.9 C	2.2 D	1.0 C	0.7 C
3 WW+LA+CH+FR	2.3 AB	2.8 B	1.8 A	1.8 A
4 CH+FR	1.8 C	2.4 CD	1.0 C	0.7 C
5 WW+FR	2.2 B	2.6 BC	1.5 B	1.2 B

[a]Means within each column bearing a common letter are not significantly different (P>0.05).
[b]Interactions of replicate x storage time and replicate x treatment were significant (P<0.05) for livers (data not shown).
[c]0 storage time was measured when samples reached 0°C.
[d]WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

Table 12. *Least squares means of log reductions of* Yersinia enterocolitica *(log CFU/sample) over 6 months of frozen storage.*

	Reductions (before-treatment minus after-treatment means) of *Y. enterocolitica* (log CFU/sample)[a]			
	Intestine	Stomach	Liver[b]	Heart
Storage Time[c]				
0	0.6 C	1.2 B	1.0 C	0.9 C
2 months	1.4 B	1.9 B	1.6 B	1.4 B
4 months	1.5 B	2.5 A	1.9 A	1.4 B
6 months	2.3 A	2.5 A	1.8 AB	1.7 A
Treatment [d]				
1 WW+LA+FR	2.1 A	2.8 A	2.0 A	2.1 A
2 FR	1.0 D	1.7 C	1.5 B	1.1 B
3 WW+LA+CH+FR	1.8 B	2.4 B	1.7 AB	1.8 A
4 CH+FR	1.0 D	1.1 D	1.2 C	1.0 B
5 WW+FR	1.4 C	2.1 B	1.5 B	1.0 B

[a]Means within each column bearing a common letter are not significantly different (P>0.05).
[b]Interactions of replicate x storage time and replicate x treatment were significant (P<0.05) for livers (data not shown).
[c]0 storage time was measured when samples reached 0°C.
[d]WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

Table 13. *Least squares means of log reductions of* Campylobacter coli *(log CFU/sample) over 6 months of frozen storage.*

	Reductions (before-treatment minus after-treatment means) of *C. coli* (log CFU/sample)[a]			
	Intestine	Stomach[b]	Liver[b]	Heart[b]
Storage Time [c]				
0	3.3 C	3.0 C	2.5 C	2.2 C
2 months	4.6 B	4.4 B	3.5 B	3.2 B
4 months	5.1 A	4.6 A	3.7 B	3.7 A
6 months	5.3 A	4.6 A	4.4 A	3.8 A
Treatment [d]				
1 WW+LA+FR	5.0 A	4.3 A	3.5 B	3.7 A
2 FR	4.5 B	3.9 B	2.9 C	3.1 BC
3 WW+LA+CH+FR	4.8 AB	4.4 A	4.1 A	3.3 B
4 CH+FR	4.4 C	3.9 B	3.4 B	3.1 BC
5 WW+FR	4.2 C	4.2 A	3.6 B	3.0 C

[a]Means within each column bearing a common letter are not significantly different (P>0.05).
[b]Interactions of replicate x storage time and replicate x treatment were significant (P<0.05) for stomachs, livers, and hearts (data not shown).
[c]0 storage time was measured when samples reached 0°C.
[d]WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

Table 14. *Least squares means of log reductions of aerobic plate counts (APCs) (log CFU/sample) over 6 months of frozen storage.*

	Reductions (before-treatment minus after-treatment means) of aerobic plate count (log CFU/sample)[a]			
	Intestine	Stomach	Liver	Heart
Storage Time[b]				
0	0.6 C	1.3 C	0.5 C	1.1 C
2 months	2.1 B	2.4 B	1.1 B	1.7 B
4 months	2.1 B	2.5 B	1.2 AB	2.0 AB
6 months	2.5 A	2.9 A	1.3 A	2.1 A
Treatment [c]				
1 WW+LA+FR	2.9 A	2.7 A	1.6 A	2.3 A
2 FR	1.3 C	2.4 AB	0.7 D	0.9 C
3 WW+LA+CH+FR	2.1 B	2.5 AB	1.3 B	2.7 A
4 CH+FR	0.8 D	1.6 C	0.7 D	1.2 C
5 WW+FR	1.9 B	2.2 B	1.0 C	1.7 B

[a]Means within each column bearing a common letter are not significantly different (P>0.05).
[b]0 storage time was measured when samples reached 0°C.
[c]WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

Table 15. *Least squares means of log reductions of* Escherichia coli *(log CFU/sample) over 6 months of frozen storage.*

	Reductions (before-treatment minus after-treatment means) of generic *E. coli* (log CFU/sample)[a]			
	Intestine	Stomach	Liver	Heart
Storage Time[b]				
0	0.7 C	1.2 C	0.6 C	1.2 C
2 months	2.6 B	2.6 B	1.7 B	2.3 B
4 months	2.6 B	2.9 B	1.8 B	2.9 A
6 months	3.1 A	3.4 A	2.1 A	2.7 A
Treatment[c]				
1 WW+LA+FR	3.5 A	3.1 A	2.2 A	2.9 A
2 FR	1.7 D	2.7 B	1.3 CD	1.5 C
3 WW+LA+CH+FR	2.6 B	2.5 BC	1.8 B	3.2 A
4 CH+FR	1.3 E	2.0 D	1.1 D	1.7 C
5 WW+FR	2.3 C	2.3 C	1.4 C	2.1 B

[a] Means within each column bearing a common letter are not significantly different (P>0.05).
[b] 0 storage time was measured when samples reached 0°C.
[c] WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

Table 16. *Least squares means of log reductions of coliforms (log CFU/sample) over 6 months of frozen storage.*

	Reductions (before-treatment minus after-treatment means) of coliforms (log CFU/sample)[a]			
	Intestine	Stomach	Liver	Heart
Storage Time[b]				
0	0.8 C	1.2 C	0.7 C	1.2 C
2 months	2.6 B	2.6 B	1.7 B	2.4 B
4 months	2.6 B	2.9 B	1.8 AB	2.9 A
6 months	3.1 A	3.5 A	2.0 A	2.8 A
Treatment [c]				
WW+LA+FR	3.5 A	3.2 A	2.2 A	2.9 B
FR	1.7 D	2.8 B	1.3 C	1.5 D
WW+LA+CH+FR	2.7 B	2.6 BC	1.7 B	3.3 A
CH+FR	1.2 E	2.0 D	1.0 D	1.7 D
WW+FR	2.3 C	2.4 CD	1.5 C	2.1 C

[a] Means within each column bearing a common letter are not significantly different (P>0.05).
[b] 0 storage time was measured when samples reached 0°C.
[c] WW: water wash (10 s, 20-30°C); LA: lactic acid spray (10 s, 40-50°C); CH: chill (4°C); FR: freeze (0°C).

APPENDIX B

MEDIA

Tryptic Soy Broth (TSB)

1. Dissolve 30.0 g TSB dehydrated media (Difco) in 1000 ml of distilled water, adding

    heat as necessary.

2. Dispense 10 ml aliquots into glass test tubes.

3. Autoclave for 15 min at 121°C and 15 psi. Store at 4°C.

0.1% Peptone Water

1. Dissolve 1.0 g dehydrated peptone media (Difco) in 1000 ml of distilled water.

2. Dispense 10 ml aliquots into glass test tubes.

3. Autoclave for 15 min at 121°C and 15 psi. Store at 4°C.

Butterfield's Buffered Phosphate *(33)*

1. Dissolve 34.0 g monopotassium phosphate (KH_2PO_4) in 500 ml distilled water.

2. Adjust pH to 7.2 using 1 N sodium hydroxide (NaOH).

3. Dilute to 1.0 liter and autoclave for 15 minutes at 121°C and 15 psi. Store as stock solution at 4°C.

4. To prepare diluent, dilute 1.25 ml of stock solution to 1.0 liter with distilled water. Autoclave for 15 minutes at 121°C and 15 psi.

Blood-Free Bolton Broth

1. Dissolve 13.8 g Bolton Broth (Oxoid, CM0983) in 500 ml distilled water.

2. Autoclave for 15 min at 121°C and 15 psi. Cool to 50°C.

3. Add 1 vial of Bolton Broth Selective Supplement (Oxoid SR0183E) containing: 10 mg cefoperazone, 10 mg vancomycin, 10 mg trimethoprim, and 25 mg cycloheximide.

4. Store at 4°C in glass screw cap bottle for up to 4 weeks.

Rifampicin-Tryptic Soy Agar (rif-TSA)

1. Dissolve 40 g TSA (Difco) in 1000 ml distilled water. Boil for 1 min.

2. Autoclave for 15 min at 121°C and 15 psi. Cool to 50°C.

3. To prepare rifampicin solution, dissolve 0.1 g rifampicin (Sigma) in 5 ml ethanol and vortex to mix thoroughly. Add to 1 liter of media to make a solution of 100 μg rifampicin per liter of TSA.

4. Pour into sterile petri plates and allow plates to solidify over night. Store at 4°C for up to 4 weeks.

Rifampicin-Yersinia Selective Agar

1. Dissolve 59.5 g Yersinia Selective Agar Base (Difco) in 1000 ml distilled water. Boil 1 min.

2. Autoclave for 15 min at 121°C and 15 psi. Cool to 50°C.

3. Add 10 ml reconstituted Yersinia Antimicrobic Supplement CN (Difco).

4. Pour into sterile petri plates and allow plates to solidify over night. Store at 4°C for up to 4 weeks.

Brucella-FBP Agar

28.0 g Brucella agar (Difco)

0.25 g ferrous sulfate

0.25 g sodium metabisulfite

0.25 g sodium pyruvate

1000 ml distilled water

Suspend the ingredients in 1000 ml of distilled water, and boil for approximately 1 min to dissolve completely. Dispense 10 ml in test tubes. Autoclave at 121°C and 15 psi for 15 min and cool to 50°C. Store at 4°C for up to 4 weeks.

Campy Line Agar (CLA) *(33)*

Ingredients:

43.0 g Brucella agar (Difco)

0.5 g ferrous sulfate, heptahydrate ($FeSO_4 \cdot 7H_2O$) (Mallinckrodt Baker, Inc.,

Phillipsburg, NJ)

0.2 g sodium bisulfate (EMD Chemicals, Gibbstown, NJ)

0.5 g sodium pyruvate (Sigma-Aldrich)

1.0 g α-ketoglutaric acid (Sigma-Aldrich)

0.6 g sodium carbonate (Sigma-Aldrich)

3.0 g yeast extract (Difco)

1000 ml distilled water

Mix ingredients in a large Erlenmeyer flask and heat to boiling. Autoclave for 15 min at

121°C and 15 psi and cool to 50°C before adding supplements.

Supplements (Sigma-Aldrich unless otherwise noted):

33.0 mg cefoperazone

100.0 mg cycloheximide

10.0 mg vancomycin

0.35 mg polymyxin B

5.0 mg trimethoprim

(continued)

Campy Line Agar (continued)

200.0 mg triphenyltetrazolium chloride-TTC

10.0 mg bovine hemin

Cefoperazone: Dissolve 1.0 g of cefoperazone in 10 ml distilled water and filter-sterilize using a 0.22 μm filter (Whatman, Dassel, Germany). Dispense into sterile centrifuge tubes (VWR) and store at -80°C. Use 0.33 ml/liter.

Cycloheximide: Dissolve 2.0 g of cycloheximide into 10 ml 50% methanol and filter-sterilize using a 0.22 μm filter. Add 0.5 ml/liter. Prepare fresh daily.

Trimethoprim/Vancomycin/Polymyxin B: Dissolve 0.1 g of trimethoprim in 20 ml of 100% ethanol in a 100-ml volumetric flask. Add 0.2 g vancomycin and 0.007 g polymyxin B, and fill to 100 ml with distilled water. Gentle heating can be used as necessary to completely dissolve supplements. Filter-sterilize using a 0.22 μm filter, dispense into sterile centrifuge tubes, and store at -80°C. Use 5 ml/liter.

Triphenyltetrazolium chloride-TTC: Dissolve 20 g of TTC in 100 ml distilled water by heating gently. Filter-sterilize using a 0.22 μm filter, dispense into sterile centrifuge tubes, and store at 4°C. Use 1 ml/liter.

Hemin: Dissolve 0.5 g hemin in 10 ml of 1N sodium hydroxide in a 100-ml volumetric flask. Add distilled water to bring final volume to 100 ml. Autoclave for 15 min at 121°C and 15 psi and store at 4°C. Use 2 ml/liter.

Pour into sterile petri plates and allow plates to solidify over night. Store at 4°C for up to 4 weeks.

Acid Test Media

1. Dissolve 180 g TSA in 4.5 liters of distilled water and boil for 1 minute. Autoclave at 121°C and 15 psi for 15 minutes and cool to 50°C.

2. Divide cooled media into 9 sterile 1000 ml flasks for pH adjustments.

3. Add 88% L-lactic acid to each flask to adjust pH to 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, and 3.0. Remove 5-10 ml at a time to monitor pH change.

4. Pour into sterile petri plates and allow to dry overnight before use.

VITA

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