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Inhibition of *Clostridium Perfringens* Growth During Extended Cooling of Cooked Uncured Roast Turkey and Roast Beef Using a Concentrated Buffered Vinegar Product and a Buffered Vinegar Product

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Uncured Roast Turkey and Roast Beef Using a Concentrated Buffered

Vinegar Product and a Buffered Vinegar Product

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ABSTRACT

Inhibition of Clostridium Perfringens Growth During Extended Cooling of Cooked Uncured Roast Turkey and Roast Beef Using a Concentrated Buffered Vinegar Product and a Buffered Vinegar Product

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This research evaluates the effectiveness of a concentrated, buffered vinegar product (CBV) and a simple buffered vinegar product (BV) for controlling *Clostridium perfringens* outgrowth during extended cooling times of ready-to-eat roast turkey and roast beef respectively. Whole turkey breasts and beef inside rounds were injected with a typical brine, then ground and mixed with CBV (0.0, 2.01, 2.70 and 3.30% wt/wt) or BV (0.0, 1.75, 2.25, and 3.75% wt/wt) and a three-strain *C. perfringens* spore cocktail to a detectable level of ca. 2-3 log CFU/g. The meat was divided into 10g portions and vacuum packaged and stored frozen until tested. The meat was cooked in a programmable water bath to 71.6°C (160.8°F) in 5 hours. The meat was then cooled exponentially with the times between 48.9°C and 12.8°C (120°F and 55°F) lasting 6, 9, 12, 15, and 18 hours for the five different cooling treatments. The cooling continued until the temperature reached 4.4°C (40°F). *C. perfringens* counts were taken at 54.4°C (130°F) and 4.4°C (40°F). At a 2.01% concentration, CBV effectively limited *C. perfringens* growth to 1-log or less up to a 9-hour cooling treatment, while 2.70 and 3.30% concentrations were effective up to the 18 hour cooling treatment. BV had an inhibitory effect on *C. perfringens* outgrowth in roast beef, but did not limit growth to 1-log or less at any concentration tested for any of the cooling treatments.

Keywords: *Clostridium perfringens*, buffered vinegar, roast turkey, roast beef

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Summary

Clostridium perfringens is a bacterium which can cause foodborne illness in humans. This organism is found most often in meat products. Because *C. perfringens* creates spores which survive cooking processes and can multiply rapidly, cooling of cooked meat must occur quickly in order to limit its growth. The USDA-FSIS has established that the cooling process for uncured meat and poultry products must occur quickly enough to limit *C. perfringens* growth to 1-log or less. Cooling large diameter roasts fast enough can be difficult, and sometimes equipment failures or over packed coolers can lead to cooling deviations. In order to mitigate the risk of *C. perfringens* growth during longer cooling periods, antimicrobials may be added. Organic acid salts have been shown to be effective at limiting *C. perfringens* growth and extending the safe cooling periods in multiple studies. However, recent shifts in consumer demand have made it necessary to explore the efficacy of naturally derived antimicrobials, often containing the same organic acid salts as have been previously researched.

Clostridium perfringens as a Foodborne Illness

C. perfringens are Gram-positive spore-forming rod-shaped non-motile anaerobic bacteria. *C. perfringens* grows between 15-50°C. Most strains grow best between 43-46°C (Brynestad and Granum, 2002). *Clostridium perfringens* is found abundantly in soils, and in the intestinal tracts of both animals and humans. Meat may become contaminated with animal feces or soils during the slaughtering process. 92% of *C. perfringens* outbreaks reported to the U.S. Centers for Disease Control between 1998 and 2010 which identified the source of the outbreak were attributed to meat and poultry products (Grass et al., 2013).

The most common form of foodborne illness due to *C. perfringens* is caused by the *C. perfringens* enterotoxin (CPE). The foodborne infection occurs when large numbers of vegetative cells are ingested. The infective dose is reported to be $>10^7$ or $>10^6$ cells (Garcia and Heredia, 2009; Brynestad and Granum, 2002). The enterotoxin produced within the cell and is released when vegetative cells lyse and sporulate (Garcia and Heredia, 2009). CPE aggravates the intestinal tract by disrupting tight junction zones between intestinal epithelial cells (Veshnyakova et al., 2012). This induces fluid and electrolyte loss from the small intestine (FDA, 2012). Diarrhea and abdominal cramps are the most common symptoms reported with *C. perfringens* illness. Most cases have a fast onset of about 16 hours after ingestion of tainted food and resolve in 12-24 hours, however, symptoms may last 1-2 weeks in the infants and elderly (FDA, 2012).

C. perfringens is a particular concern for food manufacturers because it is an organism that persists in food after cooking. The spores of *C. perfringens* easily survive and grow into vegetative cells after a cook step and multiply rapidly in warm temperatures. Most strains have a generation time of less than 20 minutes between 33 and 49°C (Byrnestad, 2002), and some enterotoxin positive strains have shown generation times as little as 7.1 minutes in ground beef between 41 and 46°C (Labbe and Huang, 1995). Many cooked products, especially larger roasts and whole muscle cuts of meat will spend considerable time during their cooling cycle in this rapid growth temperature range.

Processing Standards to Control Risk of *Clostridium perfringens* Contamination in Meat

In order to manage the risk of *C. perfringens* contamination, the USDA Food Safety Inspection Service (FSIS) has issued a performance standard for temperature stabilization of uncured cooked ready to eat beef and poultry. That standard states that “there can be… no more than 1-log10 multiplication of *Clostridium perfringens* within the product," ((9CFR 318.17(a)(2), 2014; 9CFR 381.150(a)(2), 2014). In appendix B of FSIS Compliance Guidelines for Ready to Eat Products, two cooling guidelines are offered. Option #1 is to cool the product from 54.4 to 26.6°C (130 to 80°F) in 1.5 hours or less, and cool from 26.6 to 4.4°C (80 to 40°F) in 5 hours or less. Option #2 says to cool from 48.8 to 12.8°C (120 to 55°F) in 6 hours or less, and continue cooling to 4.4°C (40°F). Producers may establish custom temperature stabilization protocols that are verified by a process authority. The authority may provide evidence for the efficacy of a process from the literature or from a challenge study (FSIS, 1999a).

Controlling Growth with Organic Acid Salts

Acetic acid, citric acid, lactic acid, acetates, citrates, and lactates in the United States are GRAS substances which are commonly found in foods (21 CFR 184, 2016). Organic acids and their salts can be effective as antimicrobials. It is generally accepted that small organic acids, such as citric and acetic acids, work as antimicrobials because they are able to cross the cell membranes of microorganisms in the neutrally charged state. When the acid transitions from a low pH environment in the food matrix where the acid is neutrally charged and crosses the microbial membrane to a higher pH environment inside the microorganism the acid dissociates and ionizes. The accumulation of ions inside the cell interferes with cellular functions and impairs growth. (Theron and Lues, 2007; Thippareddi et al., 2003). Buffering organic acids with organic acid salts shifts the equilibrium toward the undissociated neutral acid species. Buffered mixtures, therefore, have more neutral species to exert antimicrobial effect.

A review by Theron and Lues (2007) discusses several applications for using organic acids in meats as a preservative agent. Applications discussed include: rinses of lactic and acetic acid used to decontaminate whole beef carcasses from a variety of enteric bacteria; post-process application for control of *Listeria monocytogenes*; and acid dips for whole cuts of meat. Mani-Lopez et al. (2012) discussed the efficacy of multiple organic acids used for controlling and reducing *Salmonella* in meat and poultry products. Organic acids are popular antimicrobials because they are inexpensive and are generally recognized as safe (GRAS) in the United States.

Organic acids and organic acid salts have been effective at controlling the germination and growth of *C. perfringens* from spores in meats during extended cooling treatments. Extended cooling treatments are those which cool slower than the FSIS prescribed 6.5 (option 1) or 6 hour cool (option 2). Table 1 below gives the summarized findings of eight papers which specifically address the use of organic acids for extending cooling times in uncured meat and poultry products using exponential cooling curves. The treatments used include sodium citrate, sodium diacetate, calcium- sodium- and potassium lactates, and proprietary blends of lemon juice and vinegar products (as sources of citrate and acetate). While each organic acid salt has slightly different efficacy on each meat system, depending on pH or species of the meat animal, generally citrates tend to be the most effective with the lowest usage rate, with acetates and lactates requiring more on a percent-weight basis to have similar effects. Of the lactates, calcium lactate is the most effective against the germination and outgrowth of *C. perfringens* during extended cooling treatments (Velugoti et al., 2007a).

Table 1. **Compared results extended cooling of uncured meat and poultry products by organic acid salts.**

Inhibitory Compound	Usage Rate (wt/wt)	Max. Effective Cool Treatment (hours)	Meat	Brine Composition	Authors	Year
Buffered Sodium Citrate	1.00%	21	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Thippareddi et al.	2003
Buffered Sodium Citrate Sodium Diacetate	1.00%	21	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Thippareddi et al.	2003
Buffered Sodium Citrate	1.00%	21	Injected Pork	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Thippareddi et al.	2003
Buffered Sodium Citrate Sodium Diacetate	1.00%	21	Injected Pork	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Thippareddi et al.	2003
Buffered Sodium Citrate	1.00%	21	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Juneja and Thippareddi	2004a
Buffered Sodium Citrate Sodium Diacetate	1.00%	21	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Juneja and Thippareddi	2004a
Sodium Lactate	1.50%	18	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Juneja and Thippareddi	2004a
Sodium Lactate and Sodium Diacetate	3.00%	21	Roast Beef	10% water, 1.5% NaCl, 0.5% sodium triphosphate	Juneja and Thippareddi	2004a
Sodium Lactate	1.00%	15	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b
Sodium Lactate	2.00%	21	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b
Sodium Acetate	1.00%	15	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b
Sodium Acetate	2.00%	21	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b
Buffered Sodium Citrate	1.00%	21	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b

Buffered Sodium Citrate Sodium Diacetate	1.00%	18	Marinated ground turkey breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Juneja and Thippareddi	2004b
Sodium Citrate	2.00%	21	Ground Beef	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Sabah et al.	2004
Sodium Lactate	2.00%	18	Ground Beef	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Sabah et al.	2004
Calcium Lactate	2.00%	21	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Potassium Lactate	2.00%	12	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Potassium Lactate	3.00%	18	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Potassium Lactate	4.80%	21	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Sodium Lactate	2.00%	12	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Sodium Lactate	3.00%	15	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Sodium Lactate	4.80%	21	Injected Turkey Breast	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate	Velugoti et al.	2007a
Calcium Lactate	1.00%	9	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b
Potassium Lactate	2.00%	9	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b
Sodium Lactate	2.00%	9	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b
Calcium Lactate	2.00%	21	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b
Potassium Lactate	3.00%	21	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b

Sodium Lactate	3.00%	21	Injected Pork	0.85% NaCl, 0.25% potato starch, 0.2% potassium tetra pyrophosphate at 12% pump rate	Velugoti et al.	2007b
MoStatin LV (buffered lemon juice and vinegar)	1.50%	9	Roast Turkey	1.5% sea salt, 0.5% turbinado sugar	Valenzuela-Martinez et al.	2010
MoStatin LV (buffered lemon juice and vinegar)	2.50%	15	Roast Turkey	1.5% sea salt, 0.5% turbinado sugar	Valenzuela-Martinez et al.	2010
MoStatin LV (buffered lemon juice and vinegar)	3.50%	21	Roast Turkey	1.5% sea salt, 0.5% turbinado sugar	Valenzuela-Martinez et al.	2010
MoStatin V (buffered vinegar)	1.25%	9	Roast Turkey	1.5% sea salt, 0.5% turbinado sugar	Valenzuela-Martinez et al.	2010
MoStatin V (buffered vinegar)	2.50%	21	Roast Turkey	1.5% sea salt, 0.5% turbinado sugar	Valenzuela-Martinez et al.	2010
MoStatin LV1 (buffered lemon juice and vinegar)	2.00%	21	Roast Beef	0.3% sodium pyro- and polyphosphates, varying NaCl levels (1-2%)	Li et al.	2012

Recent Trends for Natural Antimicrobials

In recent years there has been increasing consumer demand for more natural products containing ingredients that sound familiar to the lay consumer. In order to provide safe meat products while utilizing more readily recognizable ingredients researchers have been investigating natural sources of conventional preservative interventions. Vinegar and lemon-vinegar combinations have been investigated by Li et al. (2012) and Valenzuela-Martinez et al. (2012) for inhibiting *C. perfringens* outgrowth in uncured meat and poultry products during cooling. Jackson et al. (2011) investigated several natural alternatives to conventional cures for frankfurters and hams, ingredients included vegetable-sourced nitrate, lemon powder (citric acid), vinegar (acetic acid), and cherry powder (ascorbic acid). King et al. (2015) investigated a tropical fruit extract, dried vinegar, lemon-vinegar blend, and a buffered vinegar blend with celery derived nitrite as an alternative and natural cure for turkey breast. McDonnell et al. (2013) conducted a study screening several natural products for their antimicrobial activity against *Listeria monocytogenes* during the refrigerated storage of ready-to-eat meat and poultry products, vinegar, vinegar blends and tea tree oil were most effective. Several other researchers have also investigated essential oils for their efficacy as antimicrobials in meat products (Jayasena and Jo, 2013). The evidence shows that many naturally-sourced antimicrobials have the potential to provide similar protection as conventional antimicrobial systems.

Common Inoculated Pack Study Methods

Inoculum

Most researchers have chosen to use a cocktail of *C. perfringens* spores in inoculated pack studies. Typically, three strains which are both fast growing and produce the *C. perfringens* enterotoxin are used. A comparison of 16 studies (Juneja et al., 1994; Sabah et al., 2003; Thippareddi et al. 2003; Sabah et al., 2004; Juneja and Thippareddi 2004a; Juneja and Thippareddi 2004b; Sanchez-Plata et al., 2005; Juneja, 2005; Juneja et al., 2006; Velugoti et al. 2007a; Velugoti et al., 2007b; Valenzuela-Martinez et al., 2010; Marquez-Gonzales et al., 2011; Li et al., 2012; Juneja et al., 2013; Kennedy et al., 2013) showed that most researchers used the same three strains, namely NCTC 8238, NCTC 8239, and NCTC 10240.

Spore Suspension Preparation

In the same comparison of 16 studies cited above, all the researchers followed a protocol developed by Juneja 1993 for the preparation of *C. perfringens* spore suspensions. The spore isolation method involves growing the organism, transferring to a sporulation medium, and then centrifuging the medium to obtain the spores in a more concentrated form. The spore culture method is explained in detail in the methods section.

Recovery Agar Media and Technique

Most commonly, *C. perfringens* is cultured with Tryptose-sulfite-cycloserine (TSC) or Shahidi-Ferguson-perfringens agar (SFP) agar. Harmon et al. (1971) created TSC as an improved culture medium over SFP because the D-cycloserine was a better selective agent than the kanamycin and polymixin present in SFP. TSC agar has been reviewed to be the best overall culture medium for enumeration compared to other culture media (Mead, 1985; DeJong et al., 2003) The FDA bacterial analytical manual for *C. perfringens* specifies the use of TSC agar with or without added egg yolk emulsion (Rhodehamel and Harmon, 2001). Many researchers have not added egg yolk emulsion into either SFP agar or TSC agar when growing *C. perfringens*, Byrne et al. (2008) determined that egg yolk emulsion in TSC did not improve growth of the organism.

According to Velugoti et al. (2007a) a dual-layer pour plate method provided greater distinction of colony formation on plates. The dual-layer pour plate method is a regular pour plate with a thin layer of agar already laid in the bottom of the petri dish and a thin overlay on top of the poured portion.

Meat Preparation and Inoculation

Meat preparation for an inoculated pack study should be designed to mimic as closely as possible the conditions encountered in a real manufacturing setting. In order to recreate plant conditions, Steele and Wright (2001) inoculated whole turkey roasts by injection and mechanically blended half roasts (2 kg) after treatment in order to get a homogenous sample. Fourteen of the sixteen studies mentioned above created a smaller model (approx. 5-10 g) by first homogenizing the sample by grinding the meat, and then vacuum sealing the meat in smaller packages to mimic the low-oxygen present in the center point of roasts. Small samples of meat make it easier to control the cook process and provide better reproducibility, but it does remove the model from the actual state of the product. Grinding meat breaks down intact muscle tissue and brings bacteria into the inner matrix of the meat rather than on the surface only. While this is appropriate for roasts made from several pieces of muscle where muscle surfaces might be in the center point of the finished roast, it may seem like a step away from the real product being studied for single whole muscle roasts. However, it has been shown that needle and needleless injection of meat can carry surface contamination into the interior of whole muscles cuts of meat

(Jefferies et al., 2012; Ray et al., 2010). Half of the sixteen studies cited above added the inoculum into a larger batch of ground meat, then mixed and divided that meat to smaller subsamples; the other half of the studies first subdivided the sample, added the inoculum directly into a subsample bag, sealed the bag, and then massaged the bag manually to achieve homogenization.

Heat Shock vs. Cook Treatment

C. perfringens spores require a shock to germinate. In commercial meats, the shock required to cause spores to germinate is provided in the form of a cooking process. In the literature, most researchers studying the germination and growth of *C. perfringens* during the cooling of cooked meats place the meat in a 70-75°C water bath up to 20 minutes and then begin the cooling process. Some variations have included heating the meat up to 60-71.1°C over the course of an hour. The most underutilized method of heat shock has been an actual cook modeled after producer derived data. In 2012, Li et al. subjected their roast beef model to a 9.75 hour cook curve to 71.1°C before beginning the cool process. In a review done by Taormina and Dorsa (2004), it is suggested that inoculated pack studies be done as close to plant parameters as possible and advocated a simulated cook as a proper heat shock treatment for a cooling study. Sudden versus gradual heat treatments influence the growth and death of *C. perfringens* during the cooking process and how *C. perfringens* grows upon cooling (Taormina and Dorsa, 2004).

Cooling Treatment

The cooling curves used by most researchers are derived from exponential formulas which emulate real cooling data observed in plants. The formulas published by Sabah et al. (2003) and Sabah et al. (2004) were used in this study and were found to closely replicate the cooling behavior observed from producer data. Challenge studies with *C. perfringens* and extended cooling times of meat products usually control the cooling process between 54.4°C, just above *C. perfringens* upper growth temperature, and 7.2°C or 4.4°C, where no growth is expected to have ceased. The controlled cooling of small lab-scale samples is typically done in a temperature-controlled water bath, where the meat packets are vacuum sealed in plastic. The vacuum packaging simulates the relatively anaerobic environment of the center point of a larger roast, and provides a thin barrier across which heat can readily transfer.

MANUSCRIPT

Introduction

Clostridium perfringens is a gram-positive, spore-forming rod-shaped bacterium, which can cause foodborne illness in humans. Outbreaks attributed to this organism most often occur in meat products, especially when prepared in large quantities that require extended cooling periods (REF). Because *C. perfringens* creates spores which survive cooking processes and can multiply rapidly, cooling of cooked meat must occur quickly in order to limit its growth. The USDA-FSIS requires that the cooling process for uncured meat and poultry products occur quickly enough to limit *C. perfringens* growth to 1-log or less. Rapid cooling of large diameter roasts can be difficult; and sometimes equipment failures or over-packed coolers can lead to cooling deviations. In order to mitigate the risk of *C. perfringens* growth during longer cooling periods, antimicrobials may be added. Organic acid salts have been shown to be effective at limiting *C. perfringens* growth and extending the safe cooling periods in multiple studies. However, growing consumer demand for clean label foods have made it necessary to explore the efficacy of naturally-derived antimicrobials, which often contain these same organic acid salts, but from natural sources. The purpose of this research is to evaluate the effectiveness of two commercially-produced, naturally-derived antimicrobials - a concentrated buffered vinegar product (CBV) and a simple buffered vinegar product (BV) - for controlling *Clostridium perfringens* outgrowth during extended cooling times of ready-to-eat roast turkey and roast beef respectively.

The FSIS sets regulations for proper temperature control of cooked meat. The FSIS has set a process *standard* which cooling processes for ready-to-eat uncured meat must follow. The FSIS standard specifies that the cooling process must not allow more than 1-log multiplication of *C. perfringens*. To help processors meet this standard, the FSIS has given two *guidelines* for the cooling of ready-to-eat uncured meat which should help processors meet the process standard. The first guideline (option #1) is to cool the product form 130°F to 80°F in 1.5 hours then from 80°F to 40°F in 5 hours. The second guideline (option #2) is to cool the product from 120°F to 55°F in 6 hours, and continue cooling to 40°F before shipping. Option 2 was selected as the model for this study to create both the control (6-hour) cooling time, and the extended cooling times which apply to the 120°F to 55°F window.

Meat producers may require extended cooling times for a variety of reasons. Some products have smaller diameters which allow for adequate cooling as measured at the center point of the product, while other products by their nature have much larger diameters which can make them very difficult to cool to target temperature within the 6-6.5 hours recommended by FSIS. Due to normal fluctuations in demand, a producer might have more product in the coolers than the coolers have refrigeration capacity to cool within the FSIS timeframes. A producer may experience equipment failure in refrigeration systems which inadvertently prolong cooling. For many reasons, a producer may wish to include preservatives in their product in order to hinder

C.perfringens growth during cooling, such that a longer cooling time would not allow greater than a 1-log multiplication of the organism, and still produce safe and compliant product.

Materials and Methods

In this study roast models were constructed by grinding up raw roast as prepared by a commercial facility and packing the ground roast into 10g, vacuum-sealed portions. The bags were then submerged in a water bath which followed a heating and cooling program which replicates the temperature profile of the cold point of roasts as measured in a commercial process. For each cooling and antimicrobial treatment combination, two 10g portions were placed in the water bath. After the cook cycle was completed, one 10g portion was removed for bacterial enumeration, at the end of the cooling treatment the second 10g portion was removed for bacterial enumeration. The difference between the two portions represented the growth during the cooling treatment.

Experimental Design. Both roast turkey and roast beef were treated with an inoculation of *C. perfringens spores* and one of four levels of antimicrobial: a zero-level control, low, medium, and high. Roast turkey was treated with CBV at 0%, 2.01%, 2.70%, and 3.30%. Roast beef was treated with BV at 0%, 1.75%, 2.25%, and 2.75%. Antimicrobial usage levels were determined by their effect on the taste of the finished product. Each antimicrobial usage level was tested with each of five different cooling rates (6, 9, 12, 15, and 18 hours) with the exception of the control which was tested only for 6, 12, and 18 hours. Each cooling rate by usage level run was replicated three times for a total of 54 runs for roast turkey, and 54 runs for roast beef. The run order within each replication was randomized.

Statistical Analysis. Three replications of each cooling regimen by antimicrobial usage level were performed. All samples were plated in duplicate with one to three countable dilutions used to calculate a mean value for individual samples. The data was analyzed using analysis of variance on usage rate, cooling regimen, temperature point and run number. The growth of *C. perfringens* was calculated with using a pseudo-Bonferroni correction for multiple comparisons giving pooled 95% confidence intervals with p=0.05.

Growth of Microorganisms. *C. perfringens* strains NCTC 8238, 8239 (University of Massachusetts Amherst, Amherst MA) and 10240 (Sigma-Aldrich, product no. RQC20106; St. Louis, MO). were used in this study. These strains were selected for this study because they are widely used in the literature and because they were isolated from foodborne outbreaks, are fast growing strains, and have a high heat tolerance they represent a worst-case scenario. The three strains were grown and maintained separately in fluid thioglycollate medium at 37°C for 18-24 hours between transfers. Spores were obtained according to procedures used by Juneja et al. (1993). All microbiological media used for this research were obtained from HiMedia Laboratories (Mumbai, India) unless otherwise stated.

Preparation of Meat Samples and Inoculation. Roast turkey and roast beef was obtained from a local processor. At the processor location, both meats were first injected and tumbled in 100 pound batches with a brine mixture containing ingredients typically used in the industry excluding the antimicrobial treatment. The meat samples were stored at 7°C and transported on ice to the lab where they were then frozen at -20°C until use (up to 6 months).

Meat was thawed overnight at 4-7°C, then ground using a KitchenAid K5 stand mixer (KitchenAid; Benton Harbor, MI) using a Chef's Choice 796 food grinder attachment and 4.5

mm plate (EdgeCraft Corp.; Avondale, PA). The ground meat was thoroughly mixed with the treatment antimicrobial to produce desired concentrations using a KitchenAid K5 stand mixer with flat beater attachment for 1 minute on setting no. 2. Equal amounts of spores suspended in water from all three strains were added to the meat to a total level of 3.7-log CFU/g as calculated from the known spore concentration of the spore stocks, and mixing was continued for an additional minute on setting no. 2. The inoculated meat was divided into 10 g portions and placed in 3 mil laminated nylon polyethylene vacuum bags (Ultrasource LLC; Kansas City, MO) with oxygen transmission rate of 50-70 cc/m^2/24hr @25°C, and water vapor transmission rate of 6-7.5 g/m^2/24hr @25°C, which were hand cut from bags of a larger dimension and sealed using a manual impulse sealer (Jores MMS-305, Technopack Corp., Sunrise, FL). After samples were placed in the small bags, they were vacuum sealed to a negative pressure of 0.085 MPa (VP 210, Vacmaster, Overland Park, KS) then flattened by a flat weight to a uniform thickness approximately 2-3 mm. All samples were frozen at -40°C up to 4 weeks, and then thawed for 18-24 hours at 4-7°C before use. Samples were prepared in batches, such that all samples for a single replication were prepared at the same time and separate from other replications. For this experiment, three replications of each cooling regimen by antimicrobial usage level combination were performed. In total

Antimicrobial Treatments. The roast turkey was treated with a concentrated buffered vinegar product (CBV) at 0% (control), 2.01%, 2.70%, and 3.30% wt/wt. Roast beef was treated with a simple buffered vinegar product (BV) at 0% (control), 1.75%, 2.25%, and 2.75% wt/wt. Both CBV and BV were provided by IsoAge Technologies (Athens, GA). Concentrations of antimicrobial were determined by their final effect on product taste. The tested concentrations are near or below the concentrations at which the flavor of the product is adversely affected.

Cook and Cool procedures. For each individual cooling time and antimicrobial treatment, two individual meat sample bags were immersed in a programmed, recirculating, heated and refrigerated water bath (Model PP15R-30-A11B, PolyScience, Niles, IL) at 7°C. According to a preliminary study, it was determined that the internal temperature of the samples did not differ more than 0.3°C from the measured bath temperature. Cooking curves were obtained from a local processor in order to simulate typical processing conditions for roast turkey, and roast beef products. The roast turkey was brought to 71.1°C (160°F) in approximately 5 hours, followed by cooling. The roast beef was brought to 57.2°C (135°F) for 37 minutes in approximately 6 hours, and then began cooling. The cook time and temperatures were chosen to produce a 7-log *salmonella* lethality to reflect industry cook processes; the roast beef is cooked for a longer period of time at a lower temperature to create a rare product. In this study, we chose to base the cooling treatments on the FSIS stabilization (cooling) guideline 2 in appendix B of the Compliance Guidelines for Cooling Heat-Treated Meat and Poultry Products (FSIS, 1999a). Option #2 is available to producers as an alternative cooling schedule which should meet the performance standard, however, it allows for a smaller margin of error. The second FSIS stabilization option was selected for this study because it would be a more abusive cooling regimen than the first option (option #1), and thus if antimicrobial treatment were sufficient with the more abusive process it would be presumably sufficient for a more conservative process.

Once samples reached the appropriate cook time/temperature, the water bath followed an exponential cooling curve which dropped from 48.9 to 12.8°C (120 to 55°F) in 6, hours and continued until it reached 4.4°C (40°F) FSIS Appendix B (FSIS, 1999a), additional extended cooling regimens were tested at 9, 12, 15, and 18 hours. The graphical representations of all programs are shown in appendix C. The time and temperature points for cooling were determined mathematically using the model used by Sabah et al. (2004a, 2004b) in the following equation:

$$T=T_{initial}\, e^{\wedge}(k_{cool}t)$$

Where T=desired temperature, $T_{initial}$=initial temperature at start of cooling, k=cooling rate and t=time (hours). The cooling rate, k, is determined by the following equation:

$$k=[ln(T2/T1)]/t_c$$

Where $T2$=final cooling temperature, $T1$=initial cooling temperature, and t_c=time in hours to cool.

Enumeration of Bacteria. After the cook process, a single 10 g meat sample bag was removed from the water bath at the beginning of the cooling process at 54.4°C (130°F) and a second 10 g sample was removed from the water bath at the end of the cooling process at 4.4°C (40°F) for enumeration. Samples were placed in a bed of ice to stop further cooking. The samples were aseptically transferred to a WhirlPak filter bag (Nasco, Fort Atkinson, WI) and homogenized (Smasher, bioMerieux, Marcy-l'Étoile, France) on fast setting with sterile 0.1% peptone for 2 minutes.

Appropriate serial dilutions of the homogenate were plated onto tryptose-sulfite-cycloserine (TSC) agar using a dual layer pour method described by Velugoti et al. (2007a). Each sample was plated in duplicate. The TSC plates were incubated anaerobically at 37°C in an anaerobic chamber (DG250, Don Whitley Scientific, Shipley, U.K.) and counted after 18-24 hours. Typical black colonies were enumerated as *C. perfringens*.

Results & Discussion

The control turkey samples with 0% CBV showed growth greater than 1-log CFU/g over the 18, 12, and even 6 hour cooling treatments. Figure 1 and table 1 below shows the average growth of each hour-concentration combination as the average difference between samples enumerated at 54.4°C (start of the cooling process) and 4.4°C (the end of the cooling process). This study showed CBV to be effective at limiting the growth of *C. perfringens* in roast turkey to <1 log for up to 18 hours at 2.70 and 3.30% concentration, and up to 9 hours at 2.01% concentration (wt/wt) with p-value 0.05.

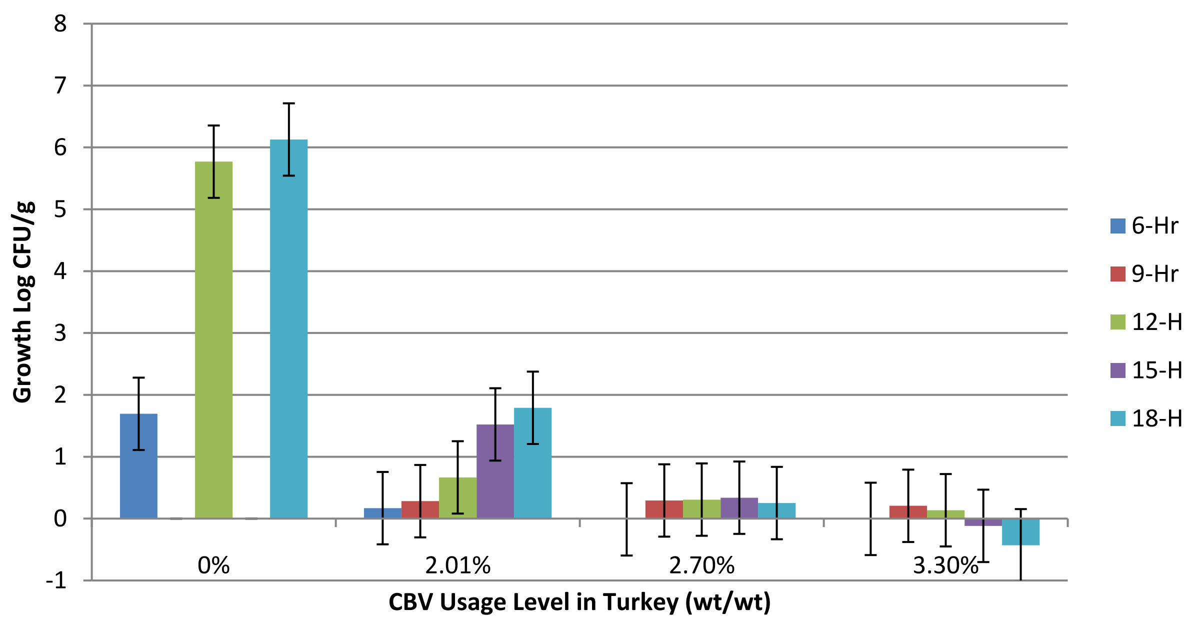

Figure 1. *C. perfringens* growth in roast turkey.

Mean growth of *C. perfringens* during the extended cooling of roast turkey with CBV. Mean growth was calculated as the difference of *C. perfringens* counts at 4.4°C from 54.4°C. Negative values indicate decreases in *C. perfringens* population. Values are shown with pooled, pseudo-Bonferroni corrected 95% confidence intervals with p=0.05, n=54.

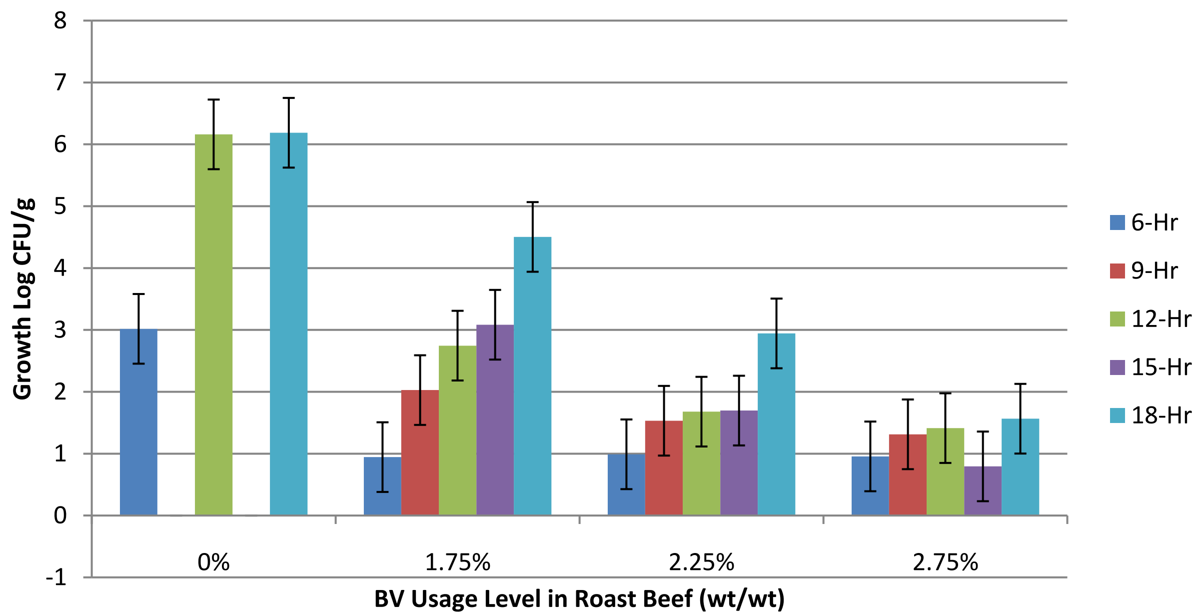

Figure 2. *C. perfringens* growth in roast beef.

Mean growth of *C. perfringens* during the extended cooling of roast beef with BV. Mean growth was calculated as the difference of *C. perfringens* counts at 4.4°C from 54.4°C. Negative values indicate decreases in *C. perfringens* population. Values are shown with pooled, pseudo-Bonferroni corrected 95% confidence intervals with p=0.05, n=54.

No combination of BV usage level and hour cooling treatment yielded <1-log growth in the roast beef (see figure 2). However, the inhibitory effect of the BV was more pronounced at higher concentrations. We did not expect the control treatment at 6 hours for either meat to fail to meet the performance standard. The FSIS Appendix B document states that the second cooling guideline offers a "significantly smaller margin of safety" than the first cooling guideline. The second guideline admonishes producers that cooling should occur as rapidly as possible, and that if cooling remains between 48.9°C (120°F) and 26.6°C (80°F) for more than one hour then compliance with the performance standard is less certain. While the wording of this guideline is certainly full of admonition to cool quickly, it does not seem to require rapid cooling to 26.6°C (80°F), it only seems to require that 48.9°C (120°F) to 12.8°C (55°F) be 6 hours or less.

According to the FSIS, the temperature range of rapid growth for *Clostridia spp*. is between 54.4°C (130°F) and 26.6°C (80°F), thus product should be cooled as rapidly as possible from 54.4°C (130°F) and 26.6°C (80°F) (FSIS, 1999b). The treatment in this experiment was to cool the product from 48.9°C (120°F) to 12.8°C (55°F) in 6 hours and continue cooling to 4.4°C (40°F). The cooling curve was generated mathematically and closely approximated the cooling observed in full sized roasts observed at a local processor. The 6-hour cooling schedule did not move quickly through the rapid growth temperature range (48.9°C (120°F) and 26.6°C (80°F)). For the 6-hour treatments, both the turkey and the roast beef remained in the rapid growth range for approximately 3 hours 15 minutes. The longer than recommended time in the rapid growth temperature range likely caused the control turkey and beef roasts to fail to meet the performance standard. From this data it seems that FSIS option #2 allows for cooling schedules which may easily allow a 1-log or greater growth of *C. perfringens*.

Initially, we were concerned that the long cook would reduce the inoculum spore count and make enumeration difficult as was shown by Shigehisa et al. (1985) using a model system of fluid thioglycollate medium. Shigehisa et al. characterized the growth of a *C. perfringens* spore culture at different heating and cooling rates. Shigehisa et al. found that at cook rates of 13°C/hour and 7°C/hr, which most closely matched our roast turkey and roast beef cook rates (approximately 14°C/hr and 9.6°C/hr), the total inoculum decreased by almost one and two log cycles respectively. Because of a relative dearth of published research subjecting spores to a cook (a slow rise to cook temperature as seen in full sized roasts) rather than a heat shock (a rapid rise to cook temperature), we decided to inoculate at a higher level of ca. 3.7 log CFU/g to compensate for a possible one log cycle reduction. The cooked samples had a mean level of 2.8 log CFU/g, which agreed with the Shigehisa study; however, when we compared our cooked sample and a heat shocked sample (20 min 75°C), both had similar levels of *C. perfringens* to each other, within 0.25 log cycles (data not shown). Both heat treatments yielded less than our calculated inoculation level based on the spore suspension counts. This discrepancy is likely due to the inability to capture all of the *C. perfringens* cells inside the matrix of the ground meat with stomacher style homogenization and not cell death in the meat matrices. Colwell style stomachers have become the preferred method of homogenization in food microbiology labs, however, other homogenization methods have shown to have even greater inner-matrix recovery of microorganisms (Rhode et al., 2015) but are limited by their inability to handle large volumes of sample. Marquez-Gonzales et al. (2012) showed that *C. perfringens* counts did not significantly change during heating of cured ground pork up to 75°C at rates of 75°C/20 min, and 12, 8, and 4°C/hr. This may explain why there was no observed difference between cooked samples and heat shocked samples.

The results of this experiment agree with previous research. Other researchers have seen effective control of *C. perfringens* using acetate sources alone (Juneja and Thippareddi, 2004b; Valenzuela-Martinez et al., 2010). The present study showed that the CBV is effective at limiting the growth of *C. perfringens* in roast turkey to less than 1-log for up to 18 hours at 2.70% and 3.30% concentration, and up to 9 hours at 2.01% concentration (wt/wt) during abusive cooling schedules. The BV used in roast beef at the concentrations tested was not effective at limiting *C perfringens* outgrowth to <1-log during any of the cooling treatments tested. The BV did have an inhibitory effect with increasing concentration and it may be effective at higher usage rates, however higher usage rates with a vinegar-based product can lead to undesirable changes in the flavor of the product. Juneja and Thippareddi (2004b) found that sodium acetate was able to safely extend cooling of marinated ground turkey breast to 15 and 21 hours at usage levels of

1.0% and 2.0% respectively. Valenzuela-Martinez et al. (2010) showed that a buffered vinegar blend was capable of inhibiting growth for 9 and 21 hour cooling treatments at usage rates of 1.25% and 2.50% respectively. The concentration of acetate ion is not known in the BV, and may be a contributing factor of why this product was unable to limit growth in the roast beef to acceptable levels. A difference between the current study and these other two studies cited is based in a difference of how the cooling process was designed. The current study set the time to cool from 48.9 to 12.8°C (120 to 55°F) in the prescribed hour treatment (6, 9, 12, 15, 18) and continued cooling until 4.4°C (40°F) was reached, whereas these two studies set the treatment time as the time to cool from 54.4°C (130°F) to 7.2°C (45°F) or sometimes 4.4°C (40°F). This difference means that more time is spent at higher temperatures during the present study than others cited herein. The fact that these other studies looked at a different product and meat species may also contribute to the difference in results.

Conclusion

C. perfringens growth is effectively inhibited by the concentrated buffered vinegar product (CBV) to less than 1-log up to 9 hours at a 2.01% usage rate and up to 18 hours with 2.70 and 3.30% usage. The simple buffered vinegar product (BV) did not effectively inhibit *C. perfringens* during extended cooling tested at the concentrations tested (1.75, 2.25, and 2.75%). Both roast turkey and roast beef controls produced more than 1-log growth of *C. perfringens* during the 6 hour cool designed to fit parameters allowed by FSIS appendix B option #2 (FSIS, 1999a). This study suggests that FSIS appendix B option #2 (FSIS, 1999a), which allows cooling of uncured ready-to-eat meat and poultry from 48.9 to 12.8°C (120 to 55°F) in 6 hours or less with continued cooling to 4.4°C (40°F) is insufficient for limiting *C.perfringens* growth to less than 1-log CFU/g during the cooling cycle, and should be revised for clarity regarding cooling during the rapid growth temperature range from 54.4°C (130°F) and 26.6°C (80°F).

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APPENDIX A: WATER BATH VALIDATION

Introduction:

The cooking and cooling curves used to program the bath come from producer data taken at the center point of large roasts. Each 10 g meat pack is to be representative of what happens at the center point of the large roasts. In order to be able to practically equate the water bath temperature and the 10 g meat pack temperature, a validation study needed to be performed.

Methods:

Overview. In this validation, thirty meat packs were placed into the water bath and they were each packed to a target of 12 g, approximately 3-4 mm thick, with bag dimensions of 4.5 cm X 11 cm. A type-T thermocouple data logger was used to gather data from four individual meat packs, and from two points in the water bath.

The parameters of this test were designed to be conservative in relation to the parameters of tests planned for the overall research project. The tests used for the whole research project would have 6-16 meat packs in the water bath at once, and the meat packs would be packed to a target of 10 g, approximately 2-3 mm thick, with bag dimensions of 4.5 cm X 11 cm.

Meat preparation. Roast beef was ground as described in the extended methods appendix B and hand packed into plastic bags and sealed. The meat was also dyed red to indicate homogeneity after mixing the meat.

Thermocouple placement. Thermocouples were inserted into four meat packs. The resulting holes in the probed meat packs were suspended above the water line, while the ends of the probes measured meat temperature that was below the water line.

Program. The water bath was programed to reach 135.5F in approximately 7 hours and cool down to 40F in approximately 11 hours.

Results:

Temperatures were taken every minute for the entire cook and cool curve tested, totaling 17 hours 54 minutes. The difference of the average water bath temperature (from the two probes in the water bath) from the average meat pack temperature (from the four meat pack probes) was shown to be very small and practically negligible over the course of a long cook and cool curve.

The average difference was -0.067°F with a standard deviation of 0.076°F. Table 4 below shows the descriptive statistics of the test, the histogram below (figure 3) shows the graphical distribution of the average pack – average water temperature differences.

Table 2. Results of water bath validation study

	Difference of Average Pack - Average Water Temperature °F	Range of Packet Temperature °F	Range of Water Temperature °F
Mean	-0.067	0.138	0.062
SD	0.076	0.103	0.040
Max.	0.300	1.230	0.180
Min.	-0.305	0.000	0.000

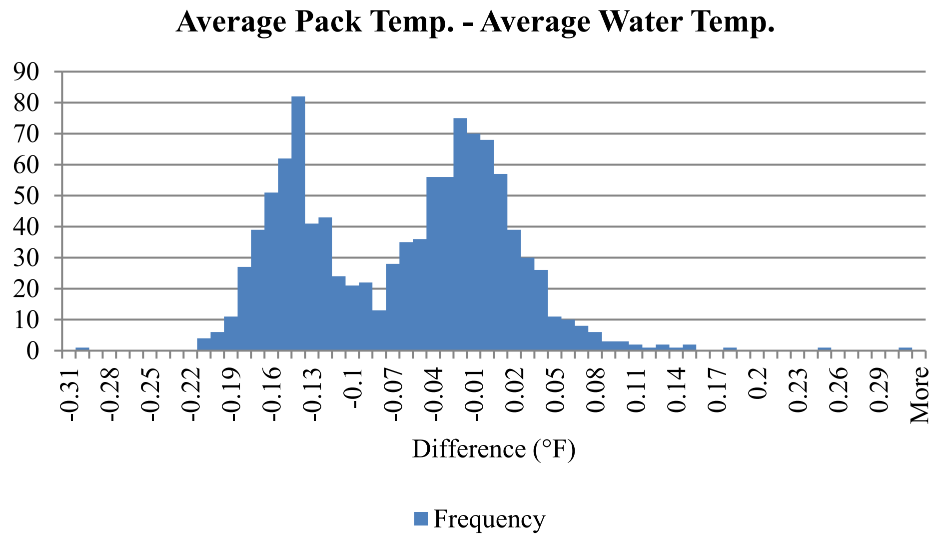


Figure 3. Histogram of water bath validation study

Conclusion:

The results of this preliminary study show that the meat pack temperature does not practically differ from the water temperature of the bath. Thus, the programmed temperature of the water bath may be considered the same temperature as the meat packs. There is no need to place thermocouple probes in a meat pack during each individual run to verify temperature.

APPENDIX B: EXTENDED METHODS

***C. perfringens* Stock Spore Preparation**

From personal email communication with Marangeli Osoria and Vijay Juneja, from the Residue Chemistry and Predictive Microbiology lab at the USDA ARS. October 16, 2015.

1. Inoculate 10ml of freshly prepared Fluid Thyoglycolate medium (FTG) with 0.1 ml of spores (2-3 tubes per strain) and incubate overnight (18 – 24h) at 37°C. [Tip: Poor growth in FTG results in poor sporulation in DS medium.]

2. Transfer overnight culture (from tube that shows the best growth) to fresh FTG tubes and incubate at 37°C for 4h.

3. Use this to inoculate the Duncan Strong Medium (DS) and incubate for 24h at 37°C. Add culture at 1% concentration to DS medium. [Tip: Consider making 1L DS per strain to generate a large amount of spores at one time that can be stored frozen.]

4. Examine spore formation using phase contrast microscopy (oil immersion 1000x; yield 90-95% spores) before harvesting. [See Procedures for Heat-Fixing Bacterial Smears and Endospore Staining sheet]

5. If spore yield appears to be less than 90%, continue to incubate the inoculated DS for another 24h and re-examine for spore formation as indicated in step 4. [Tip: I found that usually 24h is not enough for an approx. 90% spore yield, so I have incubated DS for 5-7 days and then examined spore formation.]

6. Harvest spores by centrifugation at 10000 x g for 15min at 4°C, wash twice with sterile dH2O, re-suspend in sterile dH2O and store at -20°C. [Tip: For 1L DS, you can spin down cells using

250ml sterile centrifuge bottles and wash/re-suspend with 50ml dH2O]. A working stock can be stored at 4°C until use.

7. Activate spores by heat-shocking spores at 75°C for 20min. To end heat-shock transfer tubes to an ice-water slurry for a few minutes.

8. Determine the number of spores by serially diluting heat-shocked spores in 0.1% peptone water and duplicate plating on Tryptose-sulfite- cycloserine (TSC) agar without egg yolk enrichment. Overlay plates with an additional 10ml of TSC agar and allow it to solidify before incubating plates for 48h at 37°C in an anaerobic chamber.

Recovery Medium: Tryptose-sulfite- cycloserine (TSC) = Shahidi Ferguson Perfingens (SFP) Agar Base without egg yolk enrichment + 1% D-cycloserine with overlay (10ml).

Procedures for Heat-Fixing Bacterial Smears and Endospore Staining

From personal email communication with Marangeli Osoria and Vijay Juneja, from the Residue Chemistry and Predictive Microbiology lab at the USDA ARS. October 16, 2015.

1. Heat-Fixing a Bacterial Smear
    a. Use a wax pencil to draw a circle on the microscope slide to separate each type of bacteria that will be heat-fixed.
    b. Place a drop of water onto the wax circle.
    c. Use a sterile inoculation loop and obtain a sample of a bacterial colony.
    d. Gently mix the bacteria into the water drop.
    e. Let it air dry.
    f. Pass the dried slide through the flame of a Bunsen burner or set over a hot plate with a wire mesh under the slide for a few seconds to heat-fix.
    g. Stain slide.

2. Endospore Stain Procedure
    a. Heat-fix bacterial smears.
    b. Place water in a beaker and set over a hot plate to steam.
    c. Set slides over the steaming water and apply Malachite Green (primary stain) to the smear for 5-10 minutes.
    d. Continue to apply malachite green as needed so as not to allow the stain to dry out.
    e. Rinse the slide gently with DI water.

f. Apply Safranin (counterstain; not over the steaming water) to the smear for 1-2 minutes.

g. Rinse the slide gently with DI water, blot dry and examine using phase contrast microscopy (oil immersion 1000x; yield 90-95% spores) before harvesting.

h. Vegetative cells will stain red and spores will stain blue/green.

Duncan Strong Medium Preparation Protocol

Preparation

1. Suspend 34 g of HiMedia Duncan Strong medium in 990 ml of dH2O
2. Mix thoroughly. Heat if necessary to dissolve medium completely
3. Sterilize by autoclaving for 15 minutes at 15 lbs pressure (121°C)
4. Cool to ~50°C and add 10 ml of filter-sterilized 1% caffeine solution (w/v)
    a. use syringe and 0.1 ul filter to filter sterilize

Spore Concentration Protocol

Centrifugation

1. Divide the Duncan Strong sporulation medium evenly among 250 ml sterile (autoclaved) centrifuge bottles
2. Close the centrifuge bottles **securely**
3. Take the bottles to LSB 3118 to use the Sorvall High Speed Centrifuge
    a. set temperature to 4°C
    b. Set RPM to 8120 (10,000 RCF)
    c. Set time to 20 minutes
    d. Select rotor code 28 (SLA-1500)
    e. To insert a rotor:
        i. Open door of centrifuge, latch button is on front face of machine, top right corner labelled DOOR
        ii. Place rotor into centrifuge
        iii. Insert samples into rotor
        iv. Place lid on rotor and screw counterclockwise until tightened
        v. Screw center pin counterclockwise until tightened
    f. After rotor is secure, close the door and flip the START switch
4. Centrifuge at 10,000 x g (or RCF) at 4°C for 20 minutes

Washing the Spores

5. Remove centrifuge bottles from the Centrifuge
6. Decant and properly dispose of the supernatant

7. Add 50 ml of sterile dH2O

8. Re-cap bottle **securely**

9. Shake bottle to resuspend the culture

10. Centrifuge the spores again until the spores have been washed twice

Spores Suspension

11. Follow steps 5-9

12. Combine all spores suspensions of one strain together and shake to homogenize

13. Store suspension at 4°C

Heat Shock Spore Activation

Preparation

1. Set the programmable water bath to a static 75°C
2. Prepare an ice bath in a suitable container. Obtain ice from the ice machine in the Autoclave room (S-164)

Heat Shock

3. Place cultures or sample in water bath at 75°C for 20 minutes
4. After 20 minutes on heat, place the cultures in the ice bath to cool and stop the heat shock
5. Serially dilute and plate on TSC agar by dual layer pour method to determine amount of spores per gram.

Meat Grinding and Mixing Protocol

1. Cut meat into approximately 1 inch wide strips (in order to feed into the grinder) using a knife and cutting board.
2. Continue the rest of this procedure inside the bio-safety cabinet.
3. Grind meat through 5/4.5mm plate on a KitchenAid K5 stand mixer using a Chef's Choice 796 food grinder attachment on setting no. 2.
4. Thoroughly mix ground meat with antimicrobial using KitchenAid K5 stand mixer with flat beater attachment for 1 minute on setting no. 2
5. Inoculate meat sample with 3-strain spore cocktail to roughly a 2-log level
6. Mix thoroughly as in step four above – 1 minute on setting no. 2

Tryptose Sulfite Cycloserine Agar Preparation Protocol

1. Suspend 23.5 grams of Perfringens Agar Base (T.S.C.) in 500 ml distilled water
2. Heat to boiling to dissolve the medium completely
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes
4. Cool to 50°C and add rehydrated contents of one vial of TSC Supplement (add 2 ml sterile water to contents of one vial - 200 ug cycloserine)
5. Store prepared media between 2°C and 8°C

Notes:

- Watch carefully after flask reaches 80°C (using IR thermometer)
- Screw lid on a few turns to prevent boilover loss in autoclave

Tryptose Sulfite Cycloserine Dual Layer Pour Plate Method

Preparation

1. Prepare plates by pouring a very thin layer (~5 ml) of TSC agar
    a. these may be stored for up to a week at 2 - 8°C

Dual Layer Pour Plating

1. Place 1 ml of sample onto the thin agar layer and pour ~10 ml of tempered TSC agar
2. Swirl the agar, and replace the lid
3. When the pour plates have solidified, add an additional 5 ml layer to the top of the plate
4. When the plates have solidified, incubate anaerobically at 37°C for 24 (± 2) hours

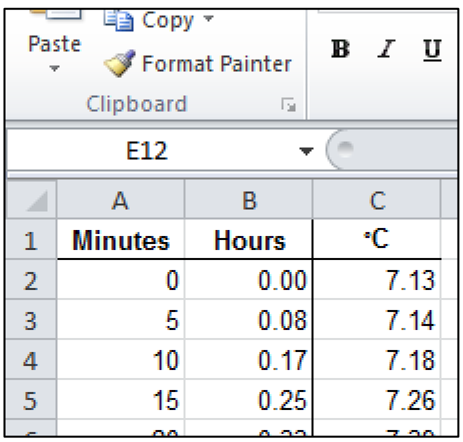
Programming the PolyScience Recirculating Water Bath

This document explains how to take time/temperature data from excel to a format which the PolyScience performance programmable water bath can utilize as a program.

Procedure:

1. <u>Prepare the Data</u>

Create an excel file with your times in one column and your temperature in another column:

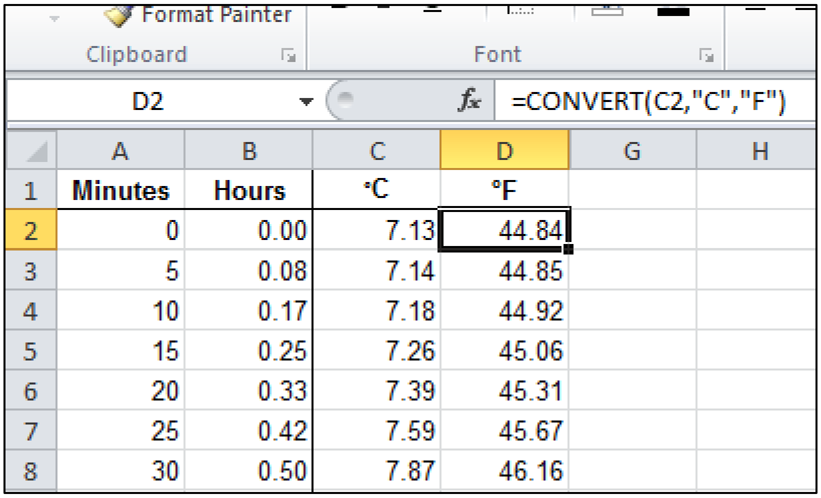


	A	B	C
1	Minutes	Hours	°C
2	0	0.00	7.13
3	5	0.08	7.14
4	10	0.17	7.18
5	15	0.25	7.26

The PolyScience program requires both Celcius and Fahrenheit values. Create another column of temperatures using the formula *=convert(reference cell,"from unit","to unit")*

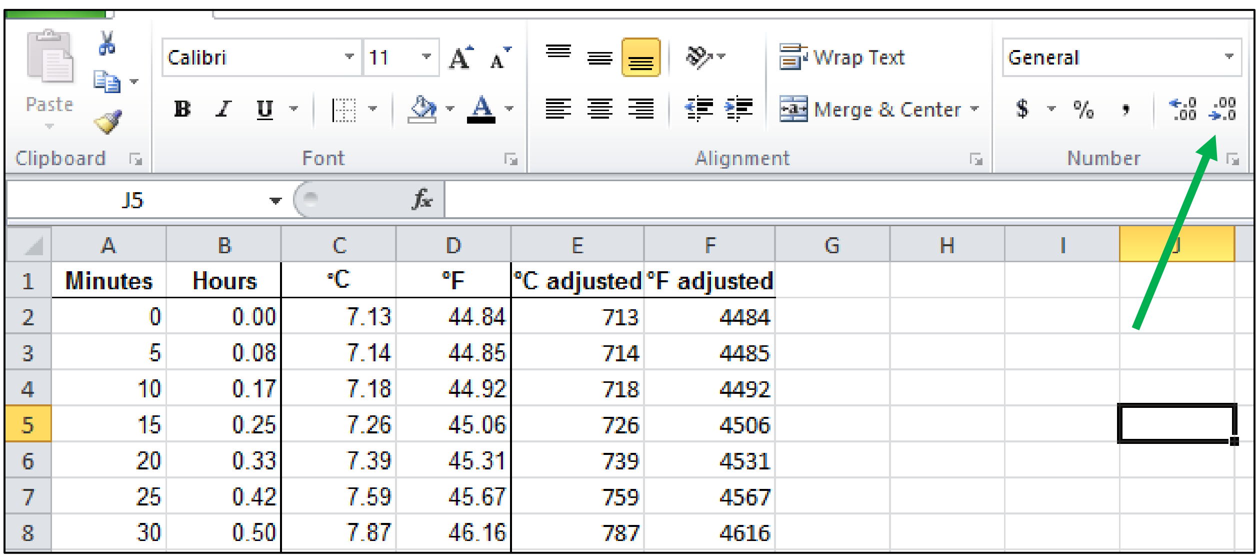
In the example case the formula is =convert(C2,"C","F")

Copy this formula to convert all your temperature units.



	Minutes	Hours	°C	°F
2	0	0.00	7.13	44.84
3	5	0.08	7.14	44.85
4	10	0.17	7.18	44.92
5	15	0.25	7.26	45.06
6	20	0.33	7.39	45.31
7	25	0.42	7.59	45.67
8	30	0.50	7.87	46.16

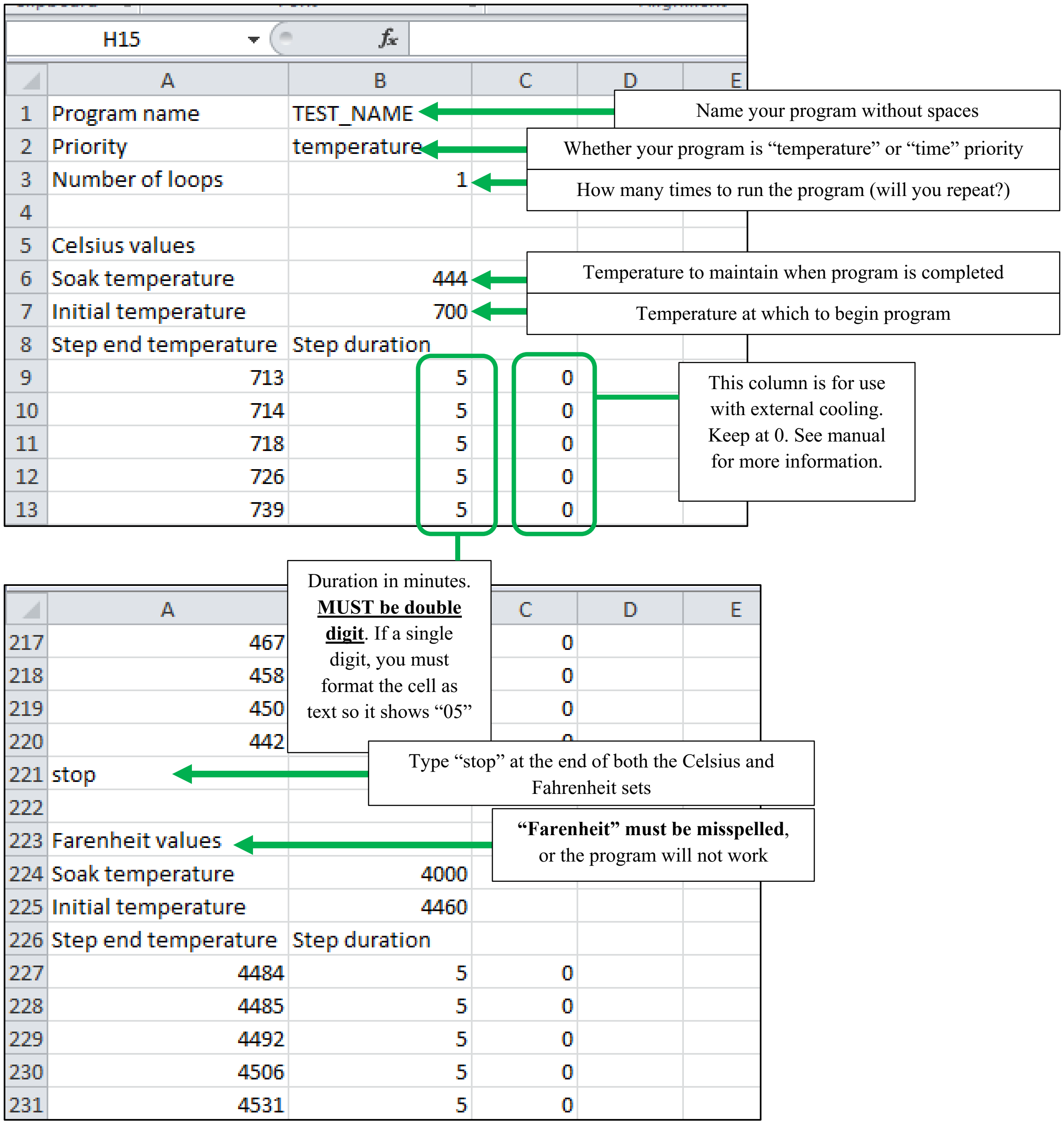
The PolyScience program will not read decimals in temperatures. Instead of 7.13°C use the number 713. To create this from your temperature columns, multiply every value by 100. Be sure to remove decimals from the value using the "decrease decimal" button.



	Minutes	Hours	°C	°F	°C adjusted	°F adjusted
2	0	0.00	7.13	44.84	713	4484
3	5	0.08	7.14	44.85	714	4485
4	10	0.17	7.18	44.92	718	4492
5	15	0.25	7.26	45.06	726	4506
6	20	0.33	7.39	45.31	739	4531
7	25	0.42	7.59	45.67	759	4567
8	30	0.50	7.87	46.16	787	4616

2. Create the Program

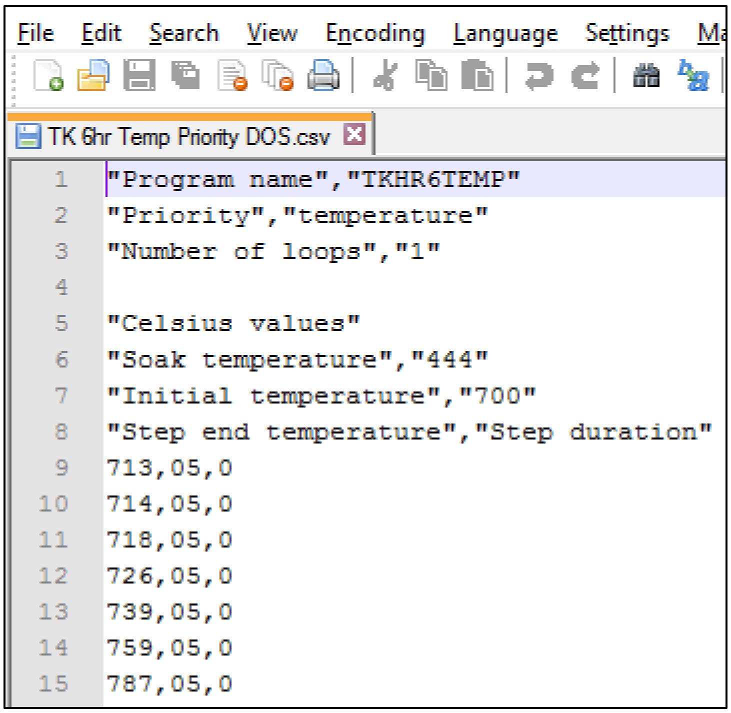
Now, create a new excel document where you will input the information in the PolyScience format. Make it look like the following:



When the file is completely assembled, save it as a .csv file.

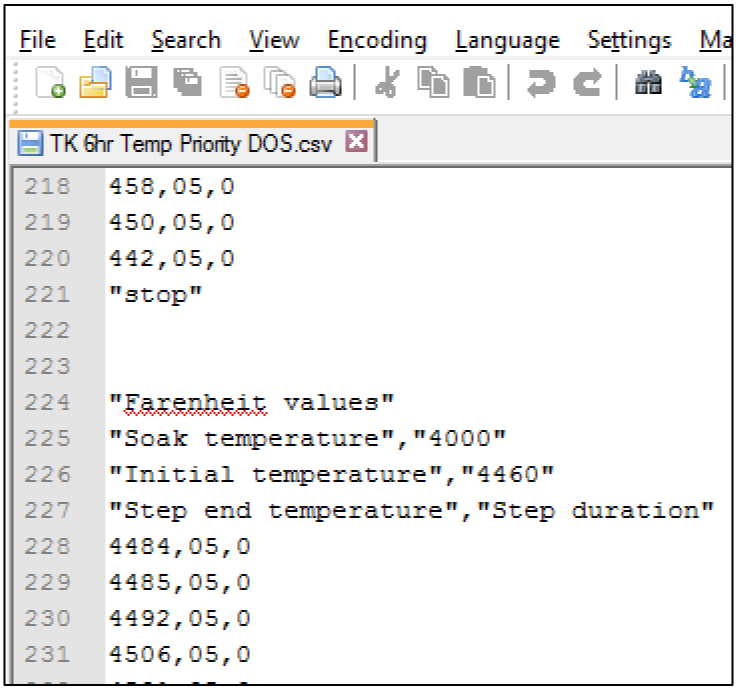
Open that file in a text editor (like *notepad* or *notepad++*).

Make your file look like the file below. It should have commas between all cells, but not between lines. Put quotes around all heading data, and the stop command at the end of the string of numbers. Then save (as .csv) and upload to the PolyScience water bath via USB drive.




```
"Program name","TKHR6TEMP"
"Priority","temperature"
"Number of loops","1"

"Celsius values"
"Soak temperature","444"
"Initial temperature","700"
"Step end temperature","Step duration"
713,05,0
714,05,0
718,05,0
726,05,0
739,05,0
759,05,0
787,05,0
```


File Edit Search View Encoding Language Settings Ma
TK 6hr Temp Priority DOS.csv
458,05,0
450,05,0
442,05,0
"stop"
"Farenheit values"
"Soak temperature","4000"
"Initial temperature","4460"
"Step end temperature","Step duration"
4484,05,0
4485,05,0
4492,05,0
4506,05,0

APPENDIX C: COMPLETE COOKING AND COOLING CURVES

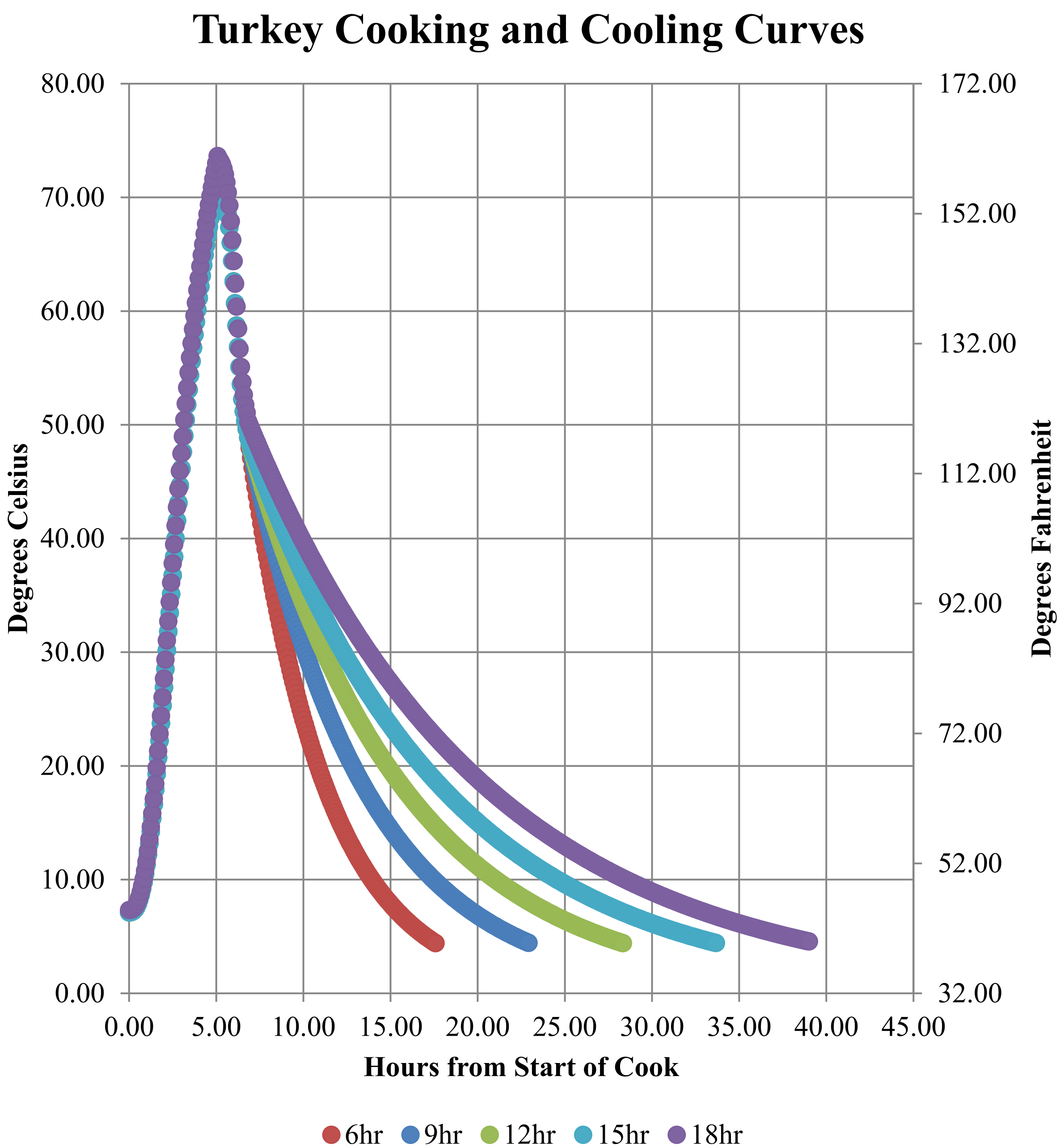


Figure 4. Roast turkey cooking and cooling curves

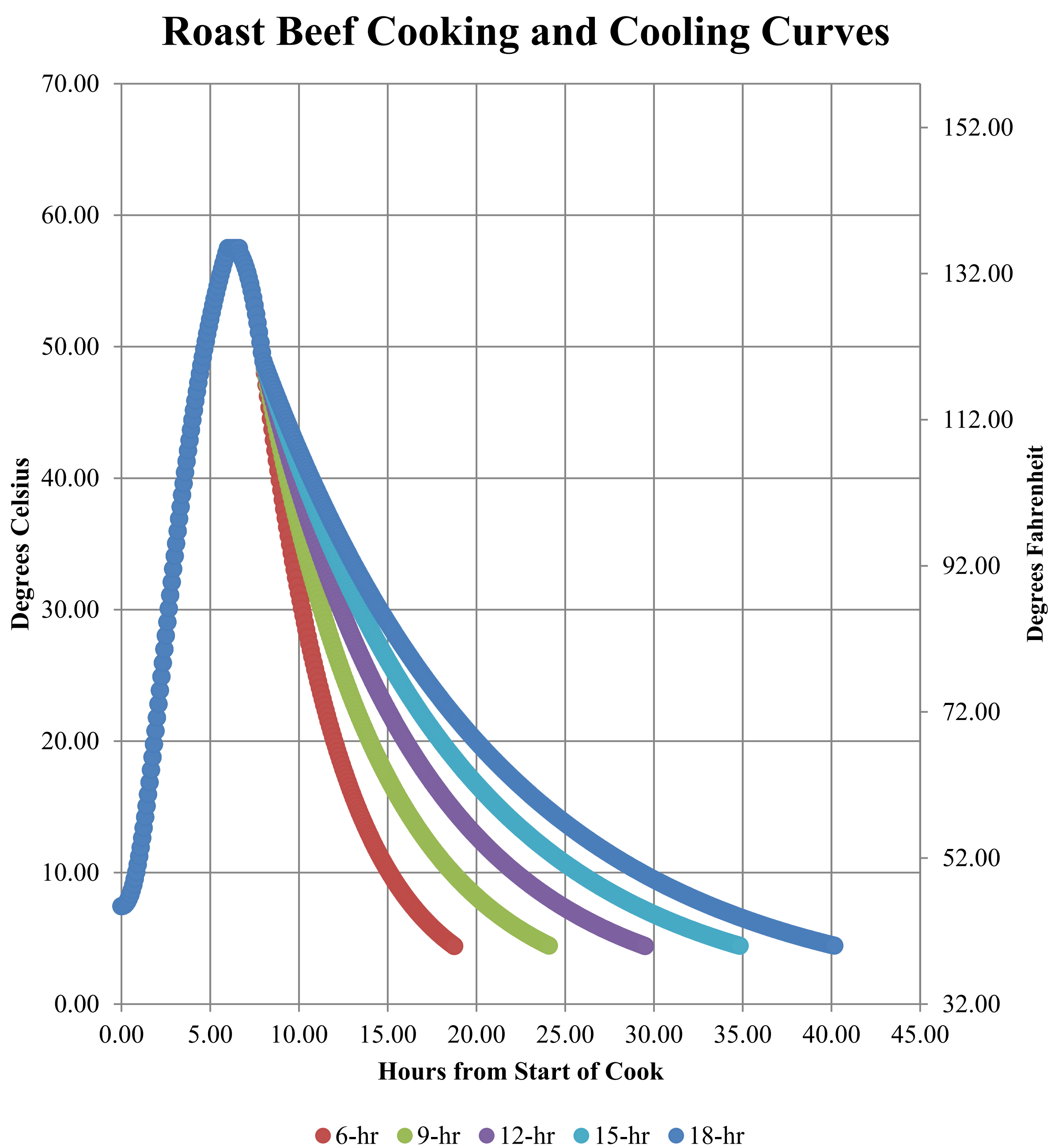


Figure 5. Roast beef cooking and cooling curves

APPENDIX D: PLATE COUNT DATA

Table 3. Raw Data: plate counts for roast turkey and roast beef

Date of Run	Meat	Usage Rate	Hour Treatment	Run (ABC)	Temperature Point	Dilution Factor																		Average CFU/g	Log_{10} CFU/g	Comments
						2	2	4	4	20	20	2E+02	2E+02	2E+03	2E+03	2E+04	2E+04	2E+05	2E+05	2E+06	2E+06	2E+07	2E+07			
3/17/2016	T	1.75	6	A	130	111	153	38	99	23	18	1	1											269	2.43	
3/17/2016	T	2.25	6	A	130	265	272	137	140	17	21	5	0											546	2.74	
3/17/2016	T	2.75	6	A	130	165	155	131	123	16	29	2	1											447	2.65	
3/17/2016	T	1.75	6	A	40	299	248	104	124	31	29	2	4											534	2.73	
3/17/2016	T	2.25	6	A	40	224	215	75	92	9	7	0	1											387	2.59	
3/17/2016	T	2.75	6	A	40	185	159	77	62	10	8	1	0											311	2.49	
3/21/2016	T	1.75	15	A	130	122	93	59	51	8	11	1	2											218	2.34	counted all colonies, black or not
3/21/2016	T	2.25	15	A	130	264	335	116	119	19	19	1	1											535	2.73	
3/21/2016	T	2.75	15	A	130	199	162	76	101	20	10	4	2											358	2.55	
3/21/2016	T	1.75	15	A	40					2703	2385	327	322											57890	4.76	White colonies appeared more on the lower dilutions. Less on the higher dilutions.
3/21/2016	T	2.25	15	A	40			624	655	147	150	23	23											2764	3.44	
3/21/2016	T	2.75	15	A	40	200	172	66	74	11	17	1	5											326	2.51	
3/23/2016	T	0	18	A	130	292	296	92	95	24	13	3	3											481	2.68	
3/23/2016	T	1.75	18	A	130	320	246	101	77	25	25	5	1											474	2.68	
3/23/2016	T	2.25	18	A	130	468	300	245	271	76	70	7	9											1087	3.04	
3/23/2016	T	2.75	18	A	130	353	839	246	487	93	107	10	13											1553	3.19	
3/23/2016	T	0	18	A	40													1952	2402	373	465			6.37E+08	8.80	
3/23/2016	T	1.75	18	A	40							1148	998	174	133	16	13							260800	5.42	
3/23/2016	T	2.25	18	A	40					18	22	3	1	0	0									400	2.60	

3/23/2016	T	2.75	18	A	40	269	221	93	95	29	21	3	4	0	0									462	2.67	
3/28/2016	T	0	12	A	130	591	530	169	157	23	29	3	2											825	2.92	
3/28/2016	T	1.75	12	A	130	803	662	294	232	14	12	2	3											1259	3.10	
3/28/2016	T	2.25	12	A	130	155	154	91	75	18	14	2	4											321	2.51	one 1/4 dilution plate had surface white growth colonies.
3/28/2016	T	2.75	12	A	130	195	212	119	112	25	21	5	3											448	2.65	
3/28/2016	T	0	12	A	40													1236	1183	96	125			231450000	8.36	
3/28/2016	T	1.75	12	A	40					440	363	50	40	6	9									8515	3.93	
3/28/2016	T	2.25	12	A	40			416	393	89	83	9	6											1669	3.22	
3/28/2016	T	2.75	12	A	40			279	241	98	66	12	5											1340	3.13	
3/29/2016	T	1.75	9	A	130	450	733	168	172	47	58													971	2.99	
3/29/2016	T	2.25	9	A	130	380	450	252	229	54	52													951	2.98	
3/29/2016	T	2.75	9	A	130	327	353	181	178	29	25													646	2.81	
3/29/2016	T	1.75	9	A	40			386	382	84	83	8	13											1603	3.20	
3/29/2016	T	2.25	9	A	40			327	304	87	70													1416	3.15	
3/29/2016	T	2.75	9	A	40			358	353	91	89													1611	3.21	
3/30/2016	T	0	6	A	130	636	362	301	470	117	94													1550	3.19	
3/30/2016	T	0	6	B	130	415	353	207	217	58	52													905	2.96	
3/30/2016	T	1.75	6	B	130	212	197	137	167	25	24													507	2.70	
3/30/2016	T	2.25	6	B	130	142	188	75	90	24	23													330	2.52	
3/30/2016	T	2.75	6	B	130	150	185	129	115	29	25													454	2.66	
3/30/2016	T	0	6	A	40							787	818											160500	5.21	
3/30/2016	T	0	6	B	40							200	171											37100	4.57	
3/30/2016	T	1.75	6	B	40	286	282	178	149	25	25													574	2.76	
3/30/2016	T	2.25	6	B	40	231	241	121	142	23	23													499	2.70	
3/30/2016	T	2.75	6	B	40	235	205	90	112	18	33													470	2.67	
3/31/2016	T	1.75	9	B	130	174	189	94	145	20	17													421	2.62	These plates were poorly swriled and the colonies are concentrated on the edges of the plate. It is difficult to

																										tell what is one colony versus multiple colonies.
3/31/2016	T	2.25	9	B	130	117	105	71	105	10	10													287	2.46	Same as above
3/31/2016	T	2.75	9	B	130	143	154	130	156	19	20													435	2.64	Same
3/31/2016	T	1.75	9	B	40			227	233	36	40	5	3											840	2.92	
3/31/2016	T	2.25	9	B	40			207	227	21	45	4	4											879	2.94	
3/31/2016	T	2.75	9	B	40			269	290	32	27	3	4											854	2.93	
4/19/2016	T	0	12	B	130			87	91	14	12													356	2.55	
4/19/2016	T	1.75	12	B	130			156	131	21	21													574	2.76	
4/19/2016	T	2.25	12	B	130			190	123	24	18													626	2.80	
4/19/2016	T	2.75	12	B	130			250	142	30	26													672	2.83	
4/19/2016	T	0	12	B	40															201	216	19	19	417000000	8.62	
4/19/2016	T	1.75	12	B	40					207	216	24	21	3	4									4230	3.63	
4/19/2016	T	2.25	12	B	40			261	236	35	34	5	6											842	2.93	
4/19/2016	T	2.75	12	B	40			227	139	20	23	3	5											732	2.86	
4/20/2016	T	0	18	B	130			312	292	37	37													974	2.99	
4/20/2016	T	1.75	18	B	130			121	96	18	13													434	2.64	
4/20/2016	T	2.25	18	B	130			199	189	25	20													684	2.84	
4/20/2016	T	2.75	18	B	130			148	189	28	19													636	2.80	
4/20/2016	T	0	18	B	40																	71	74	1450000000	9.16	
4/20/2016	T	1.75	18	B	40									6	6	0	0							12000	4.08	
4/20/2016	T	2.25	18	B	40			910	760	189	142													3325	3.52	
4/20/2016	T	2.75	18	B	40			116	79	22	9													390	2.59	
4/22/2016	T	1.75	15	B	130			135	87	16	11													444	2.65	
4/22/2016	T	2.25	15	B	130			198	162	51	44													835	2.92	
4/22/2016	T	2.75	15	B	130			214	205	39	45													839	2.92	
4/22/2016	T	1.75	15	B	40							35	43	5	2	0	0							7800	3.89	
4/22/2016	T	2.25	15	B	40			246	249	53	45	4	1											985	2.99	

4/22/2016	T	2.75	15	B	40	310	253	138	170	26	24													576	2.76	
4/22/2016	T	1.75	15	C	130			139	165	28	33													609	2.78	
4/22/2016	T	2.25	15	C	130			143	112	26	22													513	2.71	
4/22/2016	T	2.75	15	C	130			154	166	49	47													800	2.90	
4/22/2016	T	1.75	15	C	40							25	23	2	2	0	0							4800	3.68	
4/22/2016	T	2.25	15	C	40			239	239	38	40	4	5											868	2.94	
4/22/2016	T	2.75	15	C	40	163	252	184	195	27	26													568	2.75	
4/25/2016	T	0	12	C	130			234	225	31	29													759	2.88	
4/25/2016	T	1.75	12	C	130			169	158	26	22													609	2.78	
4/25/2016	T	2.25	12	C	130			117	114	18	25													475	2.68	
4/25/2016	T	2.75	12	C	130			223	215	32	31													753	2.88	
4/25/2016	T	0	12	C	40															216	204	24	28	470000000	8.67	
4/25/2016	T	1.75	12	C	40					50	72	3	5											1220	3.09	
4/25/2016	T	2.25	12	C	40			140	154	26	18													565	2.75	
4/25/2016	T	2.75	12	C	40			139	155	19	19													588	2.77	
4/26/2016	T	1.75	9	C	130			215	209	35	29													744	2.87	
4/26/2016	T	2.25	9	C	130			154	170	25	29													594	2.77	
4/26/2016	T	2.75	9	C	130			375	360	37	38													1110	3.05	
4/26/2016	T	1.75	9	C	40			433	380	84	70													1583	3.20	
4/26/2016	T	2.25	9	C	40			255	296	39	48													986	2.99	
4/26/2016	T	2.75	9	C	40			314	268	31	41													942	2.97	
4/27/2016	T	0	18	C	130			155	201	32	24													688	2.84	
4/27/2016	T	1.75	18	C	130			201	184	51	40													840	2.92	
4/27/2016	T	2.25	18	C	130			237	241	53	46													973	2.99	
4/27/2016	T	2.75	18	C	130			216	210	53	44													911	2.96	
4/27/2016	T	0	18	C	40															336	509	35	58	845000000	8.93	
4/27/2016	T	1.75	18	C	40					671	610	67	65	7	9	0	0							13005	4.11	
4/27/2016	T	2.25	18	C	40					155	153	16	13											3080	3.49	
4/27/2016	T	2.75	18	C	40			57	70	12	17													254	2.40	
4/29/2016	T	0	6	C	130			168	231	36	51													834	2.92	
4/29/2016	T	1.75	6	C	130			135	149	44	52													764	2.88	

4/29/2016	T	2.25	6	C	130			160	151	56	51													846	2.93	
4/29/2016	T	2.75	6	C	130			95	101	28	30													486	2.69	
4/29/2016	T	0	6	C	40					1157	1210													23670	4.37	
4/29/2016	T	1.75	6	C	40			285	297	56	46													1092	3.04	
4/29/2016	T	2.25	6	C	40			186	190	34	36													726	2.86	
4/29/2016	T	2.75	6	C	40			184	182	24	25													655	2.82	
6/1/2016	RB	0	12	C	130	51	70			2	7													121	2.08	
6/1/2016	RB	1.75	12	C	130	38	40			1	0													78	1.89	
6/1/2016	RB	2.25	12	C	130	15	9			2	0													24	1.38	
6/1/2016	RB	2.75	12	C	130	19	14			1	2													33	1.52	
6/1/2016	RB	0	12	C	40															72	70			142000000	8.15	
6/1/2016	RB	1.75	12	C	40							132	120											25200	4.40	
6/1/2016	RB	2.25	12	C	40					126	111													2370	3.37	
6/1/2016	RB	2.75	12	C	40	419	470			39	39													835	2.92	
6/2/2016	RB	1.75	9	C	130	29	28			7	3													57	1.76	
6/2/2016	RB	2.25	9	C	130	40	40			5	4													80	1.90	
6/2/2016	RB	2.75	9	C	130	58	60			3	5													118	2.07	
6/2/2016	RB	1.75	9	C	40	1431	1285					15	8											2716	3.43	
6/2/2016	RB	2.25	9	C	40	520	558			60	64													1159	3.06	
6/2/2016	RB	2.75	9	C	40	344	326			41	37													725	2.86	
6/3/2016	RB	0	6	C	130	159	207			9	8													366	2.56	
6/3/2016	RB	1.75	6	C	130	95	79			5	6													174	2.24	
6/3/2016	RB	2.25	6	C	130	57	43			7	2													100	2.00	
6/3/2016	RB	2.75	6	C	130	56	47			4	5													103	2.01	
6/3/2016	RB	0	6	C	40									41	49	3	6							90000	4.95	
6/3/2016	RB	1.75	6	C	40					46	53													990	3.00	
6/3/2016	RB	2.25	6	C	40					43	48													910	2.96	
6/3/2016	RB	2.75	6	C	40					34	40													740	2.87	
6/5/2016	RB	1.75	15	C	130	54	63			10	8													117	2.07	
6/5/2016	RB	2.25	15	C	130	78	77			10	6													155	2.19	
6/5/2016	RB	2.75	15	C	130	75	65			10	10													140	2.15	

6/5/2016	RB	1.75	15	C	40									260	241	24	30							534000	5.73	
6/5/2016	RB	2.25	15	C	40							51	44											9500	3.98	
6/5/2016	RB	2.75	15	C	40					94	90	11	15											1840	3.26	
6/12/2016	RB	1.75	15	E	130	268	371			26	32													610	2.78	
6/12/2016	RB	2.25	15	E	130	309	432			22	32													707	2.85	
6/12/2016	RB	2.75	15	E	130	204	362			21	34													604	2.78	
6/12/2016	RB	1.75	15	E	40									140	161	13	14							301000	5.48	
6/12/2016	RB	2.25	15	E	40					618	565	65	73											12815	4.11	
6/12/2016	RB	2.75	15	E	40					44	71	4	4											1150	3.06	
6/14/2016	RB	0	6	E	130	77	81			6	4													158	2.20	
6/14/2016	RB	1.75	6	E	130	22	24			1	0													46	1.66	
6/14/2016	RB	2.25	6	E	130	17	25			2	2													50	1.70	
6/14/2016	RB	2.75	6	E	130	18	13			0	5													31	1.49	
6/14/2016	RB	0	6	E	40									115	113	15	14							228000	5.36	
6/14/2016	RB	1.75	6	E	40	507	609			64	58													1168	3.07	
6/14/2016	RB	2.25	6	E	40	411	305			60	52													918	2.96	
6/14/2016	RB	2.75	6	E	40	318	353			45	58													851	2.93	
6/15/2016	RB	0	18	E	130	97	100			8	5													197	2.29	
6/15/2016	RB	1.75	18	E	130	61	61			6	5													122	2.09	
6/15/2016	RB	2.25	18	E	130	32	28			2	2													60	1.78	
6/15/2016	RB	2.75	18	E	130	30	46			9	9													76	1.88	
6/15/2016	RB	0	18	E	40															346	325	28	40	671000000	8.83	
6/15/2016	RB	1.75	18	E	40											293	294							5870000	6.77	
6/15/2016	RB	2.25	18	E	40							364	366											73000	4.86	
6/15/2016	RB	2.75	18	E	40					136	133													2690	3.43	
6/21/2016	RB	0	12	E	130	152	156																	308	2.49	
6/21/2016	RB	1.75	12	E	130	34	50																	84	1.92	
6/21/2016	RB	2.25	12	E	130	46	48																	94	1.97	
6/21/2016	RB	2.75	12	E	130	28	46																	74	1.87	
6/21/2016	RB	0	12	E	40													707	698	72	86			149250000	8.17	
6/21/2016	RB	1.75	12	E	40							302	324											62600	4.80	

6/21/2016	RB	2.25	12	E	40					166	160													3260	3.51	
6/21/2016	RB	2.75	12	E	40					101	101													2020	3.31	
6/22/2016	RB	1.75	9	E	130	22	14																	36	1.56	
6/22/2016	RB	2.25	9	E	130	5	18																	23	1.36	
6/22/2016	RB	2.75	9	E	130	11	7																	18	1.26	
6/22/2016	RB	1.75	9	E	40					192	219													4110	3.61	
6/22/2016	RB	2.25	9	E	40					101	97													1980	3.30	
6/22/2016	RB	2.75	9	E	40					59	52													1110	3.05	
6/26/2016	RB	1.75	15	F	130	78	71																	149	2.17	
6/26/2016	RB	2.25	15	F	130	97	67																	164	2.21	
6/26/2016	RB	2.75	15	F	130	112	119																	231	2.36	
6/26/2016	RB	1.75	15	F	40									52	66	5	8							118000	5.07	
6/26/2016	RB	2.25	15	F	40							82	99											18100	4.26	
6/26/2016	RB	2.75	15	F	40					111	112													2230	3.35	
6/28/2016	RB	0	12	F	130	189	188																	377	2.58	
6/28/2016	RB	1.75	12	F	130	50	78																	128	2.11	
6/28/2016	RB	2.25	12	F	130	56	64																	120	2.08	
6/28/2016	RB	2.75	12	F	130	22	33																	55	1.74	
6/28/2016	RB	0	12	F	40															998	1015			2013000000	9.30	
6/28/2016	RB	1.75	12	F	40							446	473											91900	4.96	
6/28/2016	RB	2.25	12	F	40					204	177													3810	3.58	
6/28/2016	RB	2.75	12	F	40					72	66													1380	3.14	
6/29/2016	RB	0	18	C	130	453	583																	1036	3.02	
6/29/2016	RB	1.75	18	C	130	148	239																	387	2.59	
6/29/2016	RB	2.25	18	C	130	147	158																	305	2.48	
6/29/2016	RB	2.75	18	C	130	154	129																	283	2.45	
6/29/2016	RB	0	18	C	40															451	565	37	27	508000172	8.71	
6/29/2016	RB	1.75	18	C	40											214	240	16	22					2270077	6.36	
6/29/2016	RB	2.25	18	C	40									126	117									121583	5.08	
6/29/2016	RB	2.75	18	C	40					305	253													5580	3.75	
6/29/2016	RB	0	18	F	130	167	177																	344	2.54	

6/29/2016	RB	1.75	18	F	130	64	90																	154	2.19	
6/29/2016	RB	2.25	18	F	130	72	93																	165	2.22	
6/29/2016	RB	2.75	18	F	130	98	70																	168	2.23	
6/29/2016	RB	0	18	F	40															340	339	48	34	749500000	8.87	
6/29/2016	RB	1.75	18	F	40											812	777	98	96					17645000	7.25	
6/29/2016	RB	2.25	18	F	40									103	127									230000	5.36	
6/29/2016	RB	2.75	18	F	40					653	537													11900	4.08	
7/3/2016	RB	0	6	F	130	188	204																	392	2.59	
7/3/2016	RB	1.75	6	F	130	133	137																	270	2.43	
7/3/2016	RB	2.25	6	F	130	105	69																	174	2.24	
7/3/2016	RB	2.75	6	F	130	121	112																	233	2.37	
7/3/2016	RB	0	6	F	40									539	663	67	62							1246000	6.10	
7/3/2016	RB	1.75	6	F	40					76	51													1270	3.10	
7/3/2016	RB	2.25	6	F	40					49	48													970	2.99	
7/3/2016	RB	2.75	6	F	40					48	39													870	2.94	
7/5/2016	RB	1.75	9	F	130	25	21																	46	1.66	
7/5/2016	RB	2.25	9	F	130	26	24																	50	1.70	
7/5/2016	RB	2.75	9	F	130	25	31																	56	1.75	
7/5/2016	RB	1.75	9	F	40					496	526													10220	4.01	
7/5/2016	RB	2.25	9	F	40					85	73													1580	3.20	
7/5/2016	RB	2.75	9	F	40					57	71													1280	3.11	

APPENDIX E: STATISTICAL OUTPUTS

Turkey Statistical Output

The SAS System 10:22 Tuesday, May 10, 2016 1137

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Log10_CFU_g
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Run__ABC_	3	A B C
Usage_Rate	4	0 1.75 2.25 2.75
Hour_Treatment	5	6 9 12 15 18
Temperature_Point	2	40 130

Dimensions

Covariance Parameters	5
Columns in X	84
Columns in Z	84
Subjects	1
Max Obs per Subject	108

Number of Observations

Number of Observations Read	108
Number of Observations Used	108
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	52.72288416	
1	4	52.77250645	.
2	3	52.58760786	0.00056910
3	1	52.56170923	0.00003746
4	1	52.56014529	0.00000021
5	1	52.56013700	0.00000000

The Mixed Procedure

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
Run__ABC_	0.000904
Run__ABC_*Usage_Rate	0
Run__ABC_*Hour_Treat	0
Run__A*Usage_*Hour_T	0
Residual	0.06940

Fit Statistics

-2 Res Log Likelihood	52.6
AIC (Smaller is Better)	56.6
AICC (Smaller is Better)	56.7
BIC (Smaller is Better)	54.8

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Usage_Rate	3	6	326.08	<.0001
Hour_Treatment	4	8	33.71	<.0001
Usage_Rat*Hour_Treat	10	20	12.93	<.0001
Temperature_Point	1	36	637.47	<.0001
Usage_Rat*Temperatur	3	36	303.84	<.0001
Hour_Trea*Temperatur	4	36	28.41	<.0001
Usage_*Hour_T*Temper	10	36	20.23	<.0001

Obs	Usage_ Rate	Hour_ Treatment	Temperature_ Point	Estimate	StdErr	Lower	Upper
1	0	6	40	4.7163	0.1531	4.3000	5.1327
2	0	6	130	3.0228	0.1531	2.6065	3.4391
3	0	12	40	8.5522	0.1531	8.1359	8.9685
4	0	12	130	2.7828	0.1531	2.3664	3.1991
5	0	18	40	8.9641	0.1531	8.5477	9.3804
6	0	18	130	2.8361	0.1531	2.4198	3.2524
7	1.75	6	40	2.8416	0.1531	2.4253	3.2580
8	1.75	6	130	2.6726	0.1531	2.2563	3.0889
9	1.75	9	40	3.1096	0.1531	2.6933	3.5259
10	1.75	9	130	2.8275	0.1531	2.4112	3.2438
11	1.75	12	40	3.5476	0.1531	3.1313	3.9639
12	1.75	12	130	2.8812	0.1531	2.4649	3.2975
13	1.75	15	40	4.1120	0.1531	3.6957	4.5283
14	1.75	15	130	2.5898	0.1531	2.1735	3.0061
15	1.75	18	40	4.5365	0.1531	4.1202	4.9528
16	1.75	18	130	2.7458	0.1531	2.3295	3.1622
17	2.25	6	40	2.7154	0.1531	2.2991	3.1317
18	2.25	6	130	2.7276	0.1531	2.3112	3.1439
19	2.25	9	40	3.0296	0.1531	2.6133	3.4459
20	2.25	9	130	2.7366	0.1531	2.3203	3.1529
21	2.25	12	40	2.9667	0.1531	2.5504	3.3830
22	2.25	12	130	2.6596	0.1531	2.2433	3.0759
23	2.25	15	40	3.1245	0.1531	2.7082	3.5408
24	2.25	15	130	2.7867	0.1531	2.3704	3.2030
25	2.25	18	40	3.2041	0.1531	2.7878	3.6204
26	2.25	18	130	2.9531	0.1531	2.5368	3.3694
27	2.75	6	40	2.6602	0.1531	2.2439	3.0765
28	2.75	6	130	2.6648	0.1531	2.2485	3.0811
29	2.75	9	40	3.0375	0.1531	2.6212	3.4538
30	2.75	9	130	2.8312	0.1531	2.4149	3.2475
31	2.75	12	40	2.9203	0.1531	2.5040	3.3366
32	2.75	12	130	2.7850	0.1531	2.3687	3.2013
33	2.75	15	40	2.6758	0.1531	2.2595	3.0921
34	2.75	15	130	2.7934	0.1531	2.3771	3.2097

35	2.75	18	40	2.5536	0.1531	2.1373	2.9699
36	2.75	18	130	2.9847	0.1531	2.5684	3.4010

Obs	Usage_ Rate	Hour_ Treatment	Estimate	StdErr	tValue	Probt	Lower	Upper
1	0	6	1.6936	0.2151	7.87	<.0001	1.1086	2.2785
70	0	12	5.7695	0.2151	26.82	<.0001	5.1845	6.3544
135	0	18	6.1280	0.2151	28.49	<.0001	5.5430	6.7129
196	1.75	6	0.1691	0.2151	0.79	0.4370	-0.4159	0.7540
253	1.75	9	0.2820	0.2151	1.31	0.1981	-0.3029	0.8670
306	1.75	12	0.6664	0.2151	3.10	0.0038	0.08147	1.2514
355	1.75	15	1.5222	0.2151	7.08	<.0001	0.9372	2.1071
400	1.75	18	1.7907	0.2151	8.32	<.0001	1.2057	2.3756
441	2.25	6	-0.01216	0.2151	-0.06	0.9552	-0.5971	0.5728
478	2.25	9	0.2930	0.2151	1.36	0.1816	-0.2919	0.8780
511	2.25	12	0.3071	0.2151	1.43	0.1620	-0.2779	0.8920
540	2.25	15	0.3378	0.2151	1.57	0.1250	-0.2471	0.9228
565	2.25	18	0.2510	0.2151	1.17	0.2508	-0.3339	0.8360
586	2.75	6	-0.00467	0.2151	-0.02	0.9828	-0.5896	0.5803
603	2.75	9	0.2064	0.2151	0.96	0.3438	-0.3786	0.7913
616	2.75	12	0.1353	0.2151	0.63	0.5333	-0.4496	0.7203
625	2.75	15	-0.1176	0.2151	-0.55	0.5880	-0.7025	0.4674
630	2.75	18	-0.4310	0.2151	-2.00	0.0526	-1.0160	0.1539

Beef Statistical Output

Obs	Usage_ Rate	Hour_ Treatment	Run__ ABC_	Temperature_ Point	Log130	Log40	log_ growth
1	0.00	6	C	40	2.56348	4.95424	2.39076
2	0.00	6	E	40	2.19866	5.35793	3.15928
3	0.00	6	F	40	2.59329	6.09552	3.50223
4	0.00	12	C	40	2.08279	8.15229	6.06950
5	0.00	12	E	40	2.48855	8.17391	5.68536
6	0.00	12	F	40	2.57634	9.30384	6.72750
7	0.00	18	C	40	3.01536	8.70586	5.69050
8	0.00	18	E	40	2.29447	8.82672	6.53226
9	0.00	18	F	40	2.53656	8.87477	6.33821
10	1.75	6	C	40	2.24055	2.99564	0.75509
11	1.75	6	E	40	1.66276	3.06744	1.40469
12	1.75	6	F	40	2.43136	3.10380	0.67244
13	1.75	9	C	40	1.75587	3.43393	1.67805
14	1.75	9	E	40	1.55630	3.61384	2.05754
15	1.75	9	F	40	1.66276	4.00945	2.34669
16	1.75	12	C	40	1.89209	4.40140	2.50931
17	1.75	12	E	40	1.92428	4.79657	2.87230
18	1.75	12	F	40	2.10721	4.96332	2.85611
19	1.75	15	C	40	2.06819	5.72754	3.65936
20	1.75	15	E	40	2.78497	5.47857	2.69359
21	1.75	15	F	40	2.17319	5.07188	2.89870
22	1.75	18	C	40	2.58771	6.35604	3.76833
23	1.75	18	E	40	2.08636	6.76864	4.68228
24	1.75	18	F	40	2.18752	7.24662	5.05910
25	2.25	6	C	40	2.00000	2.95904	0.95904
26	2.25	6	E	40	1.69897	2.96284	1.26387
27	2.25	6	F	40	2.24055	2.98677	0.74622
28	2.25	9	C	40	1.90309	3.06408	1.16099
29	2.25	9	E	40	1.36173	3.29667	1.93494
30	2.25	9	F	40	1.69897	3.19866	1.49969
31	2.25	12	C	40	1.38021	3.37475	1.99454
32	2.25	12	E	40	1.97313	3.51322	1.54009
33	2.25	12	F	40	2.07918	3.58092	1.50174

34	2.25	15	C	40	2.19033	3.97772	1.78739
35	2.25	15	E	40	2.84962	4.10772	1.25809
36	2.25	15	F	40	2.21484	4.25768	2.04283
37	2.25	18	C	40	2.48430	5.08487	2.60057
38	2.25	18	E	40	1.77815	4.86332	3.08517
39	2.25	18	F	40	2.21748	5.36173	3.14424
40	2.75	6	C	40	2.01284	2.86923	0.85639
41	2.75	6	E	40	1.49136	2.92967	1.43831
42	2.75	6	F	40	2.36736	2.93952	0.57216
43	2.75	9	C	40	2.07188	2.86034	0.78846
44	2.75	9	E	40	1.25527	3.04532	1.79005
45	2.75	9	F	40	1.74819	3.10721	1.35902
46	2.75	12	C	40	1.51851	2.92143	1.40291
47	2.75	12	E	40	1.86923	3.30535	1.43612
48	2.75	12	F	40	1.74036	3.13988	1.39952
49	2.75	15	C	40	2.14613	3.26482	1.11869
50	2.75	15	E	40	2.78104	3.06070	0.27966

Obs	Usage_ Rate	Hour_ Treatment	Run__ ABC_	Temperature_ Point	Log130	Log40	log_ growth
51	2.75	15	F	40	2.36361	3.34830	0.98469
52	2.75	18	C	40	2.45179	3.74663	1.29485
53	2.75	18	E	40	1.88081	3.42975	1.54894
54	2.75	18	F	40	2.22531	4.07555	1.85024

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Log10_CFU_g
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Run__ABC_	3	C E F
Usage_Rate	4	0 1.75 2.25 2.75
Hour_Treatment	5	6 9 12 15 18
Temperature_Point	2	40 130

Dimensions

Covariance Parameters	5
Columns in X	84
Columns in Z	84
Subjects	1
Max Obs per Subject	108

Number of Observations

Number of Observations Read	108
Number of Observations Used	108
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	72.69820093	
1	2	60.00044762	0.00000183
2	1	60.00038102	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Run__ABC_	0.004435
Run__ABC_*Usage_Rate	0
Run__ABC_*Hour_Treat	0.02285
Run__A*Usage_*Hour_T	0
Residual	0.06430

Fit Statistics

-2 Res Log Likelihood	60.0
AIC (Smaller is Better)	66.0
AICC (Smaller is Better)	66.4
BIC (Smaller is Better)	63.3

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Usage_Rate	3	6	342.84	<.0001
Hour_Treatment	4	8	30.42	<.0001
Usage_Rat*Hour_Treat	10	20	11.35	<.0001
Temperature_Point	1	36	2701.27	<.0001
Usage_Rat*Temperatur	3	36	215.43	<.0001
Hour_Trea*Temperatur	4	36	66.55	<.0001
Usage_*Hour_T*Temper	10	36	10.80	<.0001

Least Squares Means

Effect	Usage Rate	Hour Treatment	Temperature Point	Estimate	Standard Error	DF	t Value	Pr > |t|
Usage_*Hour_T*Temper	0	6	40	5.4692	0.1747	36	31.30	<.0001
Usage_*Hour_T*Temper	0	6	130	2.4518	0.1747	36	14.03	<.0001
Usage_*Hour_T*Temper	0	12	40	8.5433	0.1747	36	48.89	<.0001

Least Squares Means

Effect	Usage Rate	Hour Treatment	Temperature Point	Alpha	Lower	Upper
Usage_*Hour_T*Temper	0	6	40	0.01	4.9941	5.9444
Usage_*Hour_T*Temper	0	6	130	0.01	1.9766	2.9270
Usage_*Hour_T*Temper	0	12	40	0.01	8.0682	9.0185

The Mixed Procedure

Least Squares Means

Effect	Usage Rate	Hour Treatment	Temperature Point	Estimate	Standard Error	DF	t Value	Pr > |t|
Usage_*Hour_T*Temper	0	12	130	2.3826	0.1747	36	13.64	<.0001
Usage_*Hour_T*Temper	0	18	40	8.8025	0.1747	36	50.38	<.0001
Usage_*Hour_T*Temper	0	18	130	2.6155	0.1747	36	14.97	<.0001
Usage_*Hour_T*Temper	1.75	6	40	3.0556	0.1747	36	17.49	<.0001
Usage_*Hour_T*Temper	1.75	6	130	2.1116	0.1747	36	12.08	<.0001
Usage_*Hour_T*Temper	1.75	9	40	3.6857	0.1747	36	21.09	<.0001
Usage_*Hour_T*Temper	1.75	9	130	1.6583	0.1747	36	9.49	<.0001
Usage_*Hour_T*Temper	1.75	12	40	4.7204	0.1747	36	27.02	<.0001
Usage_*Hour_T*Temper	1.75	12	130	1.9745	0.1747	36	11.30	<.0001
Usage_*Hour_T*Temper	1.75	15	40	5.4260	0.1747	36	31.05	<.0001
Usage_*Hour_T*Temper	1.75	15	130	2.3421	0.1747	36	13.40	<.0001
Usage_*Hour_T*Temper	1.75	18	40	6.7904	0.1747	36	38.86	<.0001
Usage_*Hour_T*Temper	1.75	18	130	2.2872	0.1747	36	13.09	<.0001
Usage_*Hour_T*Temper	2.25	6	40	2.9696	0.1747	36	17.00	<.0001
Usage_*Hour_T*Temper	2.25	6	130	1.9798	0.1747	36	11.33	<.0001
Usage_*Hour_T*Temper	2.25	9	40	3.1865	0.1747	36	18.24	<.0001
Usage_*Hour_T*Temper	2.25	9	130	1.6546	0.1747	36	9.47	<.0001
Usage_*Hour_T*Temper	2.25	12	40	3.4896	0.1747	36	19.97	<.0001
Usage_*Hour_T*Temper	2.25	12	130	1.8108	0.1747	36	10.36	<.0001
Usage_*Hour_T*Temper	2.25	15	40	4.1144	0.1747	36	23.55	<.0001
Usage_*Hour_T*Temper	2.25	15	130	2.4183	0.1747	36	13.84	<.0001
Usage_*Hour_T*Temper	2.25	18	40	5.1033	0.1747	36	29.21	<.0001
Usage_*Hour_T*Temper	2.25	18	130	2.1600	0.1747	36	12.36	<.0001
Usage_*Hour_T*Temper	2.75	6	40	2.9128	0.1747	36	16.67	<.0001
Usage_*Hour_T*Temper	2.75	6	130	1.9572	0.1747	36	11.20	<.0001
Usage_*Hour_T*Temper	2.75	9	40	3.0043	0.1747	36	17.19	<.0001
Usage_*Hour_T*Temper	2.75	9	130	1.6918	0.1747	36	9.68	<.0001
Usage_*Hour_T*Temper	2.75	12	40	3.1222	0.1747	36	17.87	<.0001
Usage_*Hour_T*Temper	2.75	12	130	1.7094	0.1747	36	9.78	<.0001
Usage_*Hour_T*Temper	2.75	15	40	3.2246	0.1747	36	18.45	<.0001

Usage_*Hour_T*Temper	2.75	15	130	2.4303	0.1747	36	13.91	<.0001
Usage_*Hour_T*Temper	2.75	18	40	3.7506	0.1747	36	21.47	<.0001
Usage_*Hour_T*Temper	2.75	18	130	2.1860	0.1747	36	12.51	<.0001

Least Squares Means

Effect	Usage Rate	Hour Treatment	Temperature Point	Alpha	Lower	Upper
Usage_*Hour_T*Temper	0	12	130	0.01	1.9074	2.8577
Usage_*Hour_T*Temper	0	18	40	0.01	8.3273	9.2776
Usage_*Hour_T*Temper	0	18	130	0.01	2.1403	3.0906
Usage_*Hour_T*Temper	1.75	6	40	0.01	2.5805	3.5308
Usage_*Hour_T*Temper	1.75	6	130	0.01	1.6364	2.5867
Usage_*Hour_T*Temper	1.75	9	40	0.01	3.2106	4.1609
Usage_*Hour_T*Temper	1.75	9	130	0.01	1.1831	2.1335

The Mixed Procedure

Least Squares Means

Effect	Usage Rate	Hour Treatment	Temperature Point	Alpha	Lower	Upper
Usage_*Hour_T*Temper	1.75	12	40	0.01	4.2453	5.1956
Usage_*Hour_T*Temper	1.75	12	130	0.01	1.4994	2.4497
Usage_*Hour_T*Temper	1.75	15	40	0.01	4.9508	5.9012
Usage_*Hour_T*Temper	1.75	15	130	0.01	1.8669	2.8173
Usage_*Hour_T*Temper	1.75	18	40	0.01	6.3153	7.2656
Usage_*Hour_T*Temper	1.75	18	130	0.01	1.8120	2.7624
Usage_*Hour_T*Temper	2.25	6	40	0.01	2.4944	3.4447
Usage_*Hour_T*Temper	2.25	6	130	0.01	1.5047	2.4550
Usage_*Hour_T*Temper	2.25	9	40	0.01	2.7113	3.6616
Usage_*Hour_T*Temper	2.25	9	130	0.01	1.1794	2.1298
Usage_*Hour_T*Temper	2.25	12	40	0.01	3.0145	3.9648
Usage_*Hour_T*Temper	2.25	12	130	0.01	1.3357	2.2860
Usage_*Hour_T*Temper	2.25	15	40	0.01	3.6392	4.5895
Usage_*Hour_T*Temper	2.25	15	130	0.01	1.9431	2.8934
Usage_*Hour_T*Temper	2.25	18	40	0.01	4.6281	5.5785
Usage_*Hour_T*Temper	2.25	18	130	0.01	1.6848	2.6352
Usage_*Hour_T*Temper	2.75	6	40	0.01	2.4376	3.3880
Usage_*Hour_T*Temper	2.75	6	130	0.01	1.4820	2.4324
Usage_*Hour_T*Temper	2.75	9	40	0.01	2.5291	3.4795
Usage_*Hour_T*Temper	2.75	9	130	0.01	1.2166	2.1670
Usage_*Hour_T*Temper	2.75	12	40	0.01	2.6470	3.5974
Usage_*Hour_T*Temper	2.75	12	130	0.01	1.2342	2.1845
Usage_*Hour_T*Temper	2.75	15	40	0.01	2.7494	3.6998
Usage_*Hour_T*Temper	2.75	15	130	0.01	1.9551	2.9054
Usage_*Hour_T*Temper	2.75	18	40	0.01	3.2755	4.2258
Usage_*Hour_T*Temper	2.75	18	130	0.01	1.7108	2.6611

Obs	Usage_ Rate	Hour_ Treatment	Temperature_ Point	Estimate	StdErr	Lower	Upper
1	0	6	40	5.4692	0.1747	4.9941	5.9444
2	0	6	130	2.4518	0.1747	1.9766	2.9270
3	0	12	40	8.5433	0.1747	8.0682	9.0185
4	0	12	130	2.3826	0.1747	1.9074	2.8577
5	0	18	40	8.8025	0.1747	8.3273	9.2776
6	0	18	130	2.6155	0.1747	2.1403	3.0906
7	1.75	6	40	3.0556	0.1747	2.5805	3.5308
8	1.75	6	130	2.1116	0.1747	1.6364	2.5867
9	1.75	9	40	3.6857	0.1747	3.2106	4.1609
10	1.75	9	130	1.6583	0.1747	1.1831	2.1335
11	1.75	12	40	4.7204	0.1747	4.2453	5.1956
12	1.75	12	130	1.9745	0.1747	1.4994	2.4497
13	1.75	15	40	5.4260	0.1747	4.9508	5.9012
14	1.75	15	130	2.3421	0.1747	1.8669	2.8173
15	1.75	18	40	6.7904	0.1747	6.3153	7.2656
16	1.75	18	130	2.2872	0.1747	1.8120	2.7624
17	2.25	6	40	2.9696	0.1747	2.4944	3.4447
18	2.25	6	130	1.9798	0.1747	1.5047	2.4550
19	2.25	9	40	3.1865	0.1747	2.7113	3.6616
20	2.25	9	130	1.6546	0.1747	1.1794	2.1298
21	2.25	12	40	3.4896	0.1747	3.0145	3.9648
22	2.25	12	130	1.8108	0.1747	1.3357	2.2860
23	2.25	15	40	4.1144	0.1747	3.6392	4.5895
24	2.25	15	130	2.4183	0.1747	1.9431	2.8934
25	2.25	18	40	5.1033	0.1747	4.6281	5.5785
26	2.25	18	130	2.1600	0.1747	1.6848	2.6352
27	2.75	6	40	2.9128	0.1747	2.4376	3.3880
28	2.75	6	130	1.9572	0.1747	1.4820	2.4324
29	2.75	9	40	3.0043	0.1747	2.5291	3.4795
30	2.75	9	130	1.6918	0.1747	1.2166	2.1670
31	2.75	12	40	3.1222	0.1747	2.6470	3.5974
32	2.75	12	130	1.7094	0.1747	1.2342	2.1845
33	2.75	15	40	3.2246	0.1747	2.7494	3.6998
34	2.75	15	130	2.4303	0.1747	1.9551	2.9054

35	2.75	18	40	3.7506	0.1747	3.2755	4.2258
36	2.75	18	130	2.1860	0.1747	1.7108	2.6611

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Obs	Usage_ Rate	Hour_ Treatment	Estimate	StdErr	tValue	Probt	Lower	Upper
1	0	6	3.0174	0.2071	14.57	<.0001	2.4544	3.5805
70	0	12	6.1608	0.2071	29.75	<.0001	5.5977	6.7239
135	0	18	6.1870	0.2071	29.88	<.0001	5.6239	6.7501
196	1.75	6	0.9441	0.2071	4.56	<.0001	0.3810	1.5071
253	1.75	9	2.0274	0.2071	9.79	<.0001	1.4644	2.5905
306	1.75	12	2.7459	0.2071	13.26	<.0001	2.1828	3.3090
355	1.75	15	3.0839	0.2071	14.89	<.0001	2.5208	3.6470
400	1.75	18	4.5032	0.2071	21.75	<.0001	3.9402	5.0663
441	2.25	6	0.9897	0.2071	4.78	<.0001	0.4266	1.5528
478	2.25	9	1.5319	0.2071	7.40	<.0001	0.9688	2.0949
511	2.25	12	1.6788	0.2071	8.11	<.0001	1.1157	2.2419
540	2.25	15	1.6961	0.2071	8.19	<.0001	1.1330	2.2592
565	2.25	18	2.9433	0.2071	14.22	<.0001	2.3803	3.5064
586	2.75	6	0.9556	0.2071	4.62	<.0001	0.3926	1.5187
603	2.75	9	1.3125	0.2071	6.34	<.0001	0.7494	1.8756
616	2.75	12	1.4128	0.2071	6.82	<.0001	0.8498	1.9759
625	2.75	15	0.7943	0.2071	3.84	0.0005	0.2313	1.3574
630	2.75	18	1.5647	0.2071	7.56	<.0001	1.0016	2.1277