

## ABSTRACT

Hughes-Hollar, LaVonda Ann. In-Package Heat Pasteurization Combined with Biocide-Impregnated Packaging Films for Inhibition of *Escherichia coli* O157:H7 and *Campylobacter jejuni*.

Even with HACCP food safety programs in place, millions of pounds of ready-to-eat food products are recalled annually due to product recontamination after cooking and prior to packaging. As a consequence of this risk of contamination, numerous cases of foodborne illness occur yearly. Food processors are extremely interested in identifying and implementing additional food safety critical control points during and following packaging operations that would ensure that consumers receive products that are of high quality and that are safe.

The first objective of this study was to generate and evaluate the thermal inactivation and lethality kinetics of *E. coli* O157:H7 and *C. jejuni* inoculated on the surface of vacuum-packaged, low-fat turkey bologna slices. This investigation included determining decimal reduction times (D-values) and  $Z_D$ -values, activation energies, and rates of inactivation for these two pathogens. Packaged samples were submerged in a pre-heated water bath at temperatures of 55°, 60°, 65°, 70°, and 75°C or 53°, 55°, 60° and 62°C for *E. coli* O157:H7 and *C. jejuni*, respectively. The corresponding D-values calculated from survivor plots for *E. coli* O157:H7 were 289.5, 45.8, 15.8, 11.9 and 9.1 seconds, respectively ( $z_D$ -value = 13.9°C). D-values for *C. jejuni* at 53°, 55°, 60°, and 62°C were 272.0, 192.1, 38.4, and 25.2 seconds, respectively ( $z_D$ -value = 8.3°C). These findings indicate that *C. jejuni* has greater temperature dependence (i.e., more heat sensitive) than *E. coli* O157:H7.

The second objective of this study was to examine the inhibitory activity of a wheat gluten (70% w/w) and glycerol (30% w/w)-based film containing 500 µg/ml nisin as Nisaplin, 3% citric acid, 5 mM EDTA, and 0.50% Tween 80 (Film+N) against *E. coli*

O157:H7 and *C. jejuni* suspended in 0.1% peptone water. Alternatively, a control wheat gluten and glycerol-based film (Film) lacking nisin and the other treatment components and a 0.1% peptone water suspension without any films (Control) were tested. While the *E. coli* O157:H7 population increased 3-logs over the 72 hour exposure period (~23°C) for the Control and Film treatments, the Film+N treatment population decreased by 2-logs (a 5.6 to 7.4 log reduction). In contrast, *C. jejuni* populations decreased to below the minimum detectable level (log 1.2 cfu/ml) after 24h exposure to all three treatments. However, *C. jejuni* populations exposed to the Film+N treatment died at approximately twice the rate as the other two treatments.

Information gleaned from the previous two studies was used in designing a third study where inoculated bologna samples were subjected to a minimal in-package pasteurization process ( $\leq 1$ -log reduction) combined with the inhibitory films. A heat treatment of 60°C for 60 seconds was initially applied to the inoculated and vacuum-packaged bologna and film treatments and then stored at 4°C for 7 days. A duplicate study involving only *E. coli* O157:H7 was also conducted but the storage time was extended to 5 weeks. Minimal reductions in *E. coli* O157:H7 populations of 1.0, 0.5, and 0.35 log cfu/ml were detected across the Control, Film and Film+N treatments, respectively, following the seven day storage/exposure period. Following the extended five week refrigerated storage period, the *E. coli* O157:H7 populations declined by 1.74, 1.14 and 0.27 log cfu/ml, respectively. For *C. jejuni*, population reductions of log 1.51, 2.11, 2.68 cfu/ml were detected for the Control, Film and Film+N, respectively, following the 7-day refrigerated storage period.

The findings of this study demonstrate that wheat gluten and glycerol packaging films containing nisin, chelators and a surfactant are more inhibitory against bacterial pathogens when suspended in a liquid system as opposed to contact with a relatively dry ready-to-eat food (bologna). The loss of inhibitory activity in the combined in-package pasteurization and film treatment may be related to a lack of product moisture necessary to facilitate extraction and migration of the treatment components to the food surface. The ultimate goal of this line of investigation is to develop a modified inhibitory packaging film that would prove useful as one of several food safety “hurdles” that collectively might contribute to producing safer and higher quality food products having extended shelf lives.

**In-Package Heat Pasteurization Combined with  
Biocide-Impregnated Packaging Films for Inhibition of  
*Escherichia coli* 0157:H7 and *Campylobacter jejuni***

by

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## DEDICATION

*To Aunt Vergie, whose love and encouragement made it all possible.*

*And to my husband Ed, who made the last two years sweeter.*

## **BIOGRAPHY**

LaVonda Ann Hughes-Hollar was born on June 3, 1972 in Florence, Alabama. She attended high school at Central High School in Florence, AL. After graduating in 1990, she attended college at the University of North Alabama in Florence, AL and Southern Union Community College in Opelika, AL before enrolling at Auburn University, Auburn, AL. She earned her Bachelor of Science in Animal and Dairy Sciences (1995) and Bachelor of Science in Poultry Science in 1997. She continued her education, earning her Master of Science degree in Poultry Science at Auburn University under the direction of Dr. Donald E. Conner in 1999. She then accepted a research assistantship at North Carolina State University in pursuit of a Doctor of Philosophy degree in Food Science under the direction of Drs. Brian W. Sheldon and Patricia A. Curtis.

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## INTRODUCTION

The United States is considered to have one of the safest food systems worldwide. Yet, there are annually millions of citizens who suffer from foodborne illnesses. For example, in 2001 there were 13,705 laboratory-diagnosed cases of foodborne diseases caused by the seven bacteria and two parasites currently tracked under the FoodNet surveillance program. Due to the significant number of foodborne diseases, public health agencies, food manufacturers and food service operators alike have established goals of preventing human illness caused by foodborne pathogens. While Hazard Analysis and Critical Control Points (HACCP) programs have been established in many food processing plants to reduce the occurrence of food safety hazards, millions of pounds of ready-to-eat (RTE) products are still recalled annually. These RTE products include hotdogs, bologna and other luncheon meats that have been formulated by blending together skeletal muscles (pork, beef), mechanically deboned chicken or turkey meat, other protein sources, and ingredients such as salts, sweeteners, and cure ingredients. Although these products are thermally processed, with final internal temperatures reaching 160 to 165°F (71.1 to 73.9°C), often the product is recontaminated during the slicing, dicing, peeling processes, and packaging. The bacterial loads in commercially-produced RTE meat products can increase from  $\log_{10}$  0.5 to 2.0 cfu/g of product during the slicing process alone. Post-process handling is considered to be the primary cause of contamination of RTE meat and poultry products.

The ultimate goal of this investigation was to develop a modified inhibitory packaging film containing the inhibitory bacteriocin nisin that might prove useful as one of several food safety “hurdles” that collectively might contribute to producing safer and higher quality food products having extended shelf lives. This inhibitory peptide has received

GRAS (generally recognized as safe) status in the United States and 40 more countries as a food preservative for different food applications. Unlike many Gram positive bacteria, Gram negative bacteria are generally not sensitive to nisin. However, by combining chelating agents with nisin, its inhibitory activity can be extended to Gram negative bacteria due to a chelator-mediated change in the outer membrane permeability. Thus, to inactivate the two Gram negative pathogens of interest in this study (*Campylobacter jejuni* and *Escherichia coli* O157:H7), packaging films containing nisin, citric acid, EDTA and Tween 80 were produced and evaluated.

The initial objective of this study was to determine the thermal inactivation and lethality kinetics of *E. coli* O157:H7 and *C. jejuni* inoculated on the surface of vacuum-packaged, low-fat turkey bologna slices. This investigation included determining decimal reduction times (D-values) and  $Z_D$ -values, activation energies, and rates of inactivation. The second objective was to examine the inhibitory activity of a wheat gluten (70% w/w) and glycerol (30% w/w)-based film containing 500  $\mu\text{g/ml}$  nisin as Nisaplin, 3% citric acid, 5 mM EDTA, and 0.50% Tween 80 against *E. coli* O157:H7 and *C. jejuni* suspended in 0.1% peptone water. Information gleaned from the previous two studies was subsequently used in designing a third study where inoculated bologna samples were subjected to a minimal in-package pasteurization process combined with the inhibitory films.

## LITERATURE REVIEW

### I. Food Safety in the United States

Even though the United States has one of the safest food supplies in the world, an estimated 76 million people become sick through foodborne agents each year (Meads *et al.*, 1999). In 1995 the Foodborne Diseases Active Surveillance Network (FoodNet) began operating in five states: California, Connecticut, Georgia, Minnesota and Oregon. The purpose of FoodNet is to conduct an active surveillance for foodborne diseases and conduct epidemiology studies that will help public health officials to better understand the epidemiology of foodborne diseases in the United States. Since 1995, four additional states have been added to the surveillance network including Colorado, New York, Maryland and Tennessee. Today, 10% of the United States population, which is an estimated 25.4 million people, is under the surveillance of the FoodNet program. FoodNet actively surveys foodborne diseases caused by seven foodborne bacteria. These include diseases caused by *Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli* O157, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Vibrio* spp. (CDCP, 2002). Data collected by FoodNet from 1996-2001 showed a decrease in major bacterial foodborne illnesses, indicating that the national health objective of reducing the incidence of foodborne diseases by 2010 was occurring (USDHHS, 2000). However, more effort is still needed since foodborne illnesses in the U.S. result in an estimated 9,000 deaths and cost approximately five billion U.S. dollars annually (CAST, 1994).

In 2001, there were 13,705 laboratory-diagnosed cases of foodborne diseases caused by the seven bacteria and two parasites (*Cryptosporidium* and *Cyclospora*) tracked under FoodNet. Of these nine foodborne disease agents, *Campylobacter* was ranked second (4,470

cases) to *Salmonella* (5,198 cases) as the most common cause of confirmed bacterial gastroenteritis in 2001. Although *E. coli* O157 accounted for only 4% of the foodborne diseases, the severity of this pathogen also warrants surveillance by the Centers for Disease Control and Prevention (CDCP) (CDCP, 2002). While each of the seven bacteria is of great importance, only *Campylobacter jejuni* and *E. coli* O157:H7 will be the focus of this research investigation.

In general, foods of animal origin are more often involved in foodborne infection and intoxication (FBII) than foods of plant origin. Most likely this phenomenon occurs because animals can serve as active or passive vectors of FBII agents. In addition, animal products provide an abundance of nutrients compared to plant products. Poultry meat is considered to be the most common food to cause FBII. The pathogens most often associated with these products are *Campylobacter*, *Salmonella* and *Listeria*. While beef was once considered to be safe because the hide is removed from the edible tissue, this is no longer the case since beef-associated outbreaks of *E. coli* O157:H7 have occurred (Klinger, 2001). Once table eggs were believed to be a safe food source, but due to outbreaks of *Salmonella* associated with eggs, consumers now question their safety (CDCP, 2002).

A major goal of public health agencies, food manufacturers and food service operations is the prevention of human illnesses caused by foodborne pathogens. This goal is being achieved by the use of HACCP-based food safety principles in many U.S. and foreign food manufacturing facilities (USDA-FSIS, 1998). Other research efforts are focused on the use of active packaging systems to reduce the occurrence of foodborne diseases.

## II. *Campylobacter jejuni*

### A. History

*Campylobacter jejuni* was first reported in 1977 as a frequent cause of human bacterial diarrhea in the UK (Skirrow, 1977). An investigation from the Hospital for Infectious Diseases in Goteborg, Sweden, identified *C. jejuni* as the most common cause of bacterial diarrhea in Sweden, causing approximately 11% of the cases (Svedhem and Kaijser, 1980). Today, *C. jejuni* and *C. coli* are considered to be the most common causes of foodborne diarrhea in many industrialized nations (CPHL, 1999; Tauxe, 1992). Of all the foodborne diseases tracked by FoodNet, *Campylobacter* was ranked as the second most common cause of confirmed bacterial gastroenteritis cases (4,470 cases) in 2001; *Salmonella* was first (5,198 cases) (CDCP, 2002).

*Campylobacter*, originally known as *Vibrio fetus*, was first recognized in 1913 in infections in sheep and cattle, which led to their infertility and abortion (McFadyean and Stockman, 1913). In 1918, a similar organism was involved in a bovine abortion case in the United States (Smith, 1918). The organism was isolated from an aborted bovine fetus and was identified as *Vibrio fetus*. In subsequent years, other microaerophilic vibrios were associated with diarrhea in calves (Smith and Orcutt, 1927), bovine enteritis, winter dysentery in cattle (Jones and Little, 1931a; Jones and Little 1931b; Jones *et al.*, 1931), as well as swine dysentery (Doyle, 1944).

The first reported human outbreak involving the microaerophilic vibrios was in 1946, when approximately 350 people from an institution suffered from gastroenteritis. Twenty percent of these cases had positive fecal smears containing the microaerophilic organisms, and 13 of the 39 blood cultures were positive. It was believed that these vibrios were *Vibrio*

*jejuni* (Levy, 1946), which had been implicated in the earlier bovine cases (Jones and Little, 1931a; Jones and Little, 1931b). Other microaerophilic vibrios, believed to be *V. fetus*, were isolated from blood cultures of pregnant women who had aborted during the course of febrile illness (Vinzent *et al.*, 1947; Vinzent, 1949). In 1957, E.O. King suggested that *Vibrio fetus*, which she referred to as “related vibrios,” may be causing human illness and therefore may be an important zoonotic disease. King also recognized the difficulty in recovering the “related vibrios” from fecal samples due to their unique growth requirements (King, 1957).

Due to the significant biochemical and physiological differences between these “related vibrios” and traditional *Vibrio* spp., as well as the DNA base pair ratio differences, Sebald and Veron proposed the generic term “campylobacter” to describe the “related vibrios.” Campylobacter is derived from the Greek word for “curved rod” (Sebald and Veron, 1963) which describes the shape of the “related vibrios.” In 1973 the first comprehensive account of the taxonomy of the genus *Campylobacter* was published (Veron and Chatelain, 1973). Despite previous difficulties in isolating the bacteria, Bokkenheuser published a procedure for isolating the bacteria from animal fecal samples that involved passing the fecal suspension through a 0.65  $\mu\text{m}$  filter and then incubating the filter under microaerophilic conditions on selective agar (Bokkenheuser, 1970). Skirrow applied these same procedures to human stool samples and demonstrated that, as King had suggested earlier, *Campylobacter jejuni* was indeed an important cause of human gastroenteritis (Skirrow, 1977).

In 1980, the World Health Organization stated that *C. jejuni* infections were indeed common but believed the organism played a greater role as the cause of diarrheal disease in developing countries than in developed countries (WHO, 1980). However, *C. jejuni* has

been reported as the most common bacteria associated with acute human gastrointestinal infections in the U.S. (Blaser *et al.*, 1983), Canada (Coles *et al.*, 1985; Harvey and Greenwood, 1983), Sweden (Svedhem and Kaijser, 1980), Scotland (Brunton and Heggie, 1977) and England (Bruce *et al.*, 1977).

In addition to *C. jejuni*, there are at least two other species of *Campylobacter* that cause gastroenteritis in humans, *Campylobacter coli* and *Campylobacter laridis* (Benjamin *et al.*, 1983). *C. coli* is responsible for three to five percent of campylobacter-related human gastroenteritis cases (Karmali *et al.*, 1983; Skirrow and Benjamin, 1980a; Skirrow and Benjamin, 1982). Since the illnesses caused by *C. jejuni* and *C. coli* are indistinguishable (Karmali *et al.*, 1983; Skirrow and Benjamin, 1980a;) and the two organisms have the same optimal growth temperature (Benjamin *et al.*, 1983), it was not until 1980 that these two species could be distinguished from each another. *C. jejuni* is able to hydrolyze hippurate, while *C. coli* lacks this ability (Harvey, 1980; Hwang and Ederer, 1975; Skirrow and Benjamin, 1980b). Furthermore, *C. coli* strains are resistant to 2,3,5 triphenyltetrazolium while *C. jejuni* strains are usually not resistant. *C. jejuni* and *C. coli* are distinguished from *C. laridis* through their nalidixic acid sensitivity. *C. jejuni* and *C. coli* are both sensitive to nalidixic acid (40 mg/l or a 30 µg nalidixic acid disc) and are resistant to cephalothin (64 mg/l or a 30 µg cephalothin disc) (Karmali *et al.*, 1980; Karmali *et al.*, 1983), whereas *C. laridis* is a nalidixic acid resistant thermophilic *Campylobacter* (NARTRC) (Benjamin *et al.*, 1983).

## **B. Disease characterization**

Symptoms of campylobacteriosis are seen two to five days after consumption of contaminated product. Infectious doses can be as low as 500-800 organisms (Robinson,

1981). The illness may persist up to 10 days (Blaser *et al.*, 1979; Butzler and Skirrow, 1979). Usually the disease starts with a fever, often over 40°C, with malaise, abdominal pains and headaches, followed by diarrhea. The stools will become liquid to watery and often contain polymorphonuclear leukocytes (Blaser *et al.*, 1979). Acute diarrhea may last from two to three days with abdominal discomfort lasting for several days longer (Butzler and Skirrow, 1979). Some patients have had such severe abdominal pains due to *C. jejuni* enteritis that they were mistakenly admitted to the hospital for acute appendicitis (Skirrow, 1977) and cholecystitis (Mertens and De Smet, 1979). In mild cases the organism can be isolated only up to a few days after the patient ceases to show symptoms, yet the organism has been isolated from feces of patients two to seven weeks after symptoms have subsided in more severe cases (Anders *et al.*, 1981). Diagnosis is often determined by direct phase-contrast microscopic examination of a stool sample since large numbers of the organisms are excreted (Karmali and Fleming, 1979). While the illness is most often self-limiting and needs no antibiotic, erythromycin is prescribed in serious cases (Blaser and Reller, 1981; Pai *et al.*, 1983). Most strains of *C. jejuni* are resistant to penicillin G, cephalosporin, colistin and trimethoprium. Some *C. jejuni* strains are resistant to erythromycin and tetracycline (Vanhoof *et al.*, 1978).

*C. jejuni* has been associated with extraintestinal diseases such as cholecystitis (Darling *et al.*, 1979), urinary tract infections (Davis and Penfold, 1979), reactive arthritis (Ebright and Ryan, 1983; Rynes *et al.*, 1984; Urman *et al.*, 1977), Reiter's syndrome (Saari and Kauranen, 1980) and bacteremia (Walder *et al.*, 1982). There have been links between infections of particular serotypes of *C. jejuni* and the development of Guillain-Barre Syndrome (GBS) (Nachamkin *et al.*, 1998; Rhodes and Tattersfield, 1982). While most

illnesses associated with *C. jejuni* are uncomplicated enteritis, some deaths have occurred (Smith and Blaser, 1985).

While *Campylobacter* species are fastidious under laboratory conditions, the fact remains that these bacteria are the main cause of bacterial diarrhea in humans. How the organism causes disease is not fully understood, yet certain factors are known. The outer membrane of these Gram negative bacteria will protect the cytoplasmic membrane from exposure to bile salts of the digestive system that would otherwise lyse the cells (Inouye, 1979). The outer membrane also plays a role in adherence to the host cells as well as invasion of the host cells (McCoy *et al.*, 1975; Smith, 1977). As with all Gram negative organisms, the outer membrane lipopolysaccharides (LPS) of *C. jejuni* have endotoxin properties (Austen and Trust, 1982) that may be directly involved in toxicity of the organism (Shands, 1975). Disease may also be caused by destruction of host cells, allowing penetration through the intestinal mucosa by cytotoxin enzyme (Prescot *et al.*, 1981; Ruiz-Palacios, 1981).

### **C. Bacterial characterization**

#### **1. Morphology**

The *Campylobacter* genus has morphological and physiological similarities to the genus *Spirillum*; therefore, it is classified as a member of the Spirillaceae family (Smibert, 1984). *Campylobacter* spp. are small, spirally curved, non-spore forming Gram negative rods with a polar flagellum at one or both ends of the cell. Cell length varies from 0.5 to 0.8  $\mu\text{m}$  with widths of 0.2 to 0.5  $\mu\text{m}$  (Veron and Chatelain, 1973; Smibert, 1974; Smibert, 1984). These are highly motile organisms with movement characterized as rapid, darting, corkscrew-like motility (Neill *et al.*, 1979).

## 2. Temperature requirements

*Campylobacter* spp. are commensals in most animals, normally colonizing the gastrointestinal tracts of many different animals (CPHL, 1999; Tauxe, 1992). They are thermophilic organisms with a growth range from 34° to 44°C and an optimal growth temperature of 42°C, which would reflect an adaptation to the intestines of warm-blooded birds (Rettig, 1979). In the laboratory, *C. jejuni* isolation is improved on solid media when samples are incubated at 42°C versus 37°C (Goossens *et al.*, 1984; Janssen and Helstad, 1982; Luechetfeld *et al.*, 1982; Smibert, 1984; Wang *et al.*, 1983). This is due, in part, to the inhibition of competing microorganisms at 42°C (Goossens *et al.*, 1984). *C. jejuni* ATCC 2948 grown under 6% O<sub>2</sub> in Brucella broth at 37°C and 42°C have nearly identical growth curves, suggesting that growth of *C. jejuni* is only improved at 42°C on solid media (Lee *et al.*, 1988).

## 3. Biochemistry

*Campylobacter* spp. are considered to be fastidious organisms, requiring complex growth media. They are unable to ferment or oxidize carbohydrates (van Vliet and Kelly, 2001; Veron and Chatelain, 1973). Amino acids and tricarboxylic acid cycle intermediates are their principle energy sources (Smibert, 1974; Smibert, 1984; Veron and Chatelain, 1973). The organisms are catalase and oxidase positive and urease negative (CPHL, 1999; Tauxe, 1992). Moreover, *C. jejuni* reduces nitrates but will not hydrolyze gelatin or urea (Rettig, 1979). The DNA G and C content is between 29-36 mol% (Veron and Chatelain, 1973).

## 4. Atmospheric requirements

*Campylobacter* is highly sensitive to low levels of hydrogen peroxide and superoxide radicals in culture media (Hoffman *et al.*, 1979). Supplements commonly used in media for

*C. jejuni*, such as ferrous sulfate, sodium pyruvate, blood and charcoal, do not serve as enrichment agents but as detoxifying agents, quenching hydrogen peroxide and superoxide radicals (Krieg and Hoffman, 1986) and preventing accumulation of photochemically generated toxic oxygen derivatives (Bolton *et al.*, 1984; Hoffman *et al.*, 1979). Bowdre *et al.*, (1976) suggested that the *C. jejuni* microaerophilic requirement results from the organism's inability to synthesize ferric iron-binding compounds at a level sufficient to support aerobic growth. The aerotolerance of some *Campylobacter* spp. was increased when 0.25% ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP) were added to agar media. While there is no physiological change in *C. jejuni* when the FBP supplement is added, it is theorized that the FBP may act as an oxygen scavenger or scavenge oxygen by-products such as peroxides and superoxides (George *et al.*, 1978; Hoffman *et al.*, 1979). Furthermore, Stern *et al.*, (1985) reported that the addition of ferrous sulfate increased the recoverability of *C. jejuni* by 10,000 fold.

Finally, several other factors have been reported to inhibit *Campylobacter* growth under laboratory conditions. Nutrient agar stored in a lighted environment and under normal atmospheric conditions for 48 hours will become inhibitory to *C. jejuni* (Bolton *et al.*, 1984). For this reason, freshly prepared media are recommended, and if this is not possible, plates should be stored at 4°C under anaerobic conditions (Fricker, 1985). Also, inhibitory action of bacteriocins from *Pseudomonas aeruginosa* against some strains of *Campylobacter* has been reported (Blankwell *et al.*, 1982).

## 5. Recovery from foods

Since this organism grows at temperatures between 30°C and 45°C, it is not likely to grow in raw foods of animal origin held at proper refrigeration temperatures (Stern and

Kazmi, 1989). While the organism may not grow under refrigeration, it survives well in foods held at refrigeration temperature (Koidis and Doyle, 1984). The organism will die more quickly in foods held at room temperature than when stored at 4°C (Blankenship and Craven, 1982; Doyle and Roman, 1981). A 1- to 2-log reduction in *C. jejuni* will occur in chicken meat held for 17 days at 4°C, yet a 2.5- to 5-log reduction will occur in the same amount of time if the chicken meat is held at 23°C (Blankenship and Craven, 1982). Furthermore, *C. jejuni* is less likely to be isolated from frozen foods than from fresh foods of the same type. Fresh meat and poultry products were more likely (12.1% positive) to have *Campylobacter* spp. than the same type of frozen food product (2.3% positive) (Stern *et al.*, 1984).

To determine the occurrence of *C. jejuni*, chicken was purchased from three grocery stores. *C. jejuni* was isolated from six of the ten frozen carcasses tested, whereas five of the eight fresh carcasses (stored at 4°C) tested positive. All nine samples of minced meats (pork and beef) tested positive for *C. jejuni*. When the food products were stored at 4°C, the number of positive samples remained approximately the same when held for at least seven days. If the storage temperature was increased to 42°C, the number of positive samples increased considerably during the first day of sampling, especially in chicken, yet this was followed by a rapid decrease in positive samples with no *C. jejuni* recovered after two days of storage. When the products were stored at 20°C, a rapid decrease in the number of positive samples occurred, with no isolation of *C. jejuni* occurring after three days of storage (Svedhem *et al.*, 1981).

## 6. Viable-but-non-culturable state

Though normally spiral shaped, on exposure to atmosphere oxygen levels or other stresses, *Campylobacter* spp. have been reported to change into coccoid forms. Bacteria with the coccoid forms are considered to be in a viable-non-culturable (VNC) stage. This stage is believed to be a dormant state required for survival of the organism under these conditions (Rollins and Colwell, 1986). However, the existence and infectivity of VNC *Campylobacter* is controversial (Cappelier *et al.*, 1999; Hazeleger, 1998; Jones *et al.*, 1991; Medema *et al.*, 1992; Tholozan *et al.*, 1999). While *C. jejuni* may be non-culturable by direct plating, there could still be  $10^6$  cfu/ml of viable organisms potentially capable of colonizing and infecting a host (Rollins and Colwell, 1986).

## 7. Heat sensitivity

While the temperature for optimal growth of *C. jejuni* is 42°C, thermal inactivation occurs at 48°C. The heat sensitivity of 13 strains of clinical *Campylobacter* isolates was tested. The organisms were grown in broth under a microaerobic environment for two days at 42°C before placing five ml of each into separate glass tubes in a water bath. Using blood agar plates, 0.1 ml of the heat-treated samples were cultured and incubated at 42°C under microaerobic conditions and examined for the recovery of viable *C. jejuni*. When the samples were heat treated at 57.5°C for 45 minutes or at 60°C for longer than 15 minutes, no *C. jejuni* were recovered (Svedhem *et al.*, 1981).

D-values of *C. jejuni* in milk, ground chicken, and ground beef are summarized in Table 1. While whole milk products typically have a greater heating requirement than skim milk products (Koidis and Doyle, 1984), minimum pasteurization requirements would be sufficient to eliminate *C. jejuni* in both products (Palumbo, 1984).

**TABLE 1. D-values of *C. jejuni* in skim milk, ground chicken and beef.**

<b>Product</b>	<b>Temperature</b>	<b>D-value</b>	<b>Source</b>
Skim milk	48°C	7.2-12.8 min	Koidis and Doyle, 1983
Skim milk	55°C	0.74-1.0 min	Koidis and Doyle, 1983
Ground chicken	49°C	20 min	Blankenship and Craven, 1982
Ground chicken	57°C	45 sec	Blankenship and Craven, 1982
Ground beef	50°C	6 min	Koidis and Doyle, 1983
Ground beef	58°C	15 sec	Koidis and Doyle, 1983

### **III. *Escherichia coli* O157:H7**

#### **A. History**

*Escherichia coli* O157:H7 was established as a human pathogen in 1975 when it was isolated from a patient suffering with profuse diarrhea. While *E. coli* O157 accounted for only 4% of the confirmed cases of bacterial gastroenteritis in the US in 2001 (CDCP, 2002), it is nevertheless of great concern since this pathogen causes an estimated 20,000 cases of foodborne illnesses each year resulting in approximately 250 deaths, in addition to being the most common cause of hemolytic-uremic syndrome (HUS) in the U.S. (Altekruse *et al.*, 1997). In 2000 it was estimated that 62,458 reported cases involving *E. coli* O157:H7 cost an estimated \$659.1 million (approximately \$10,500/case) (USDA-FSIS, 2001).

#### **B. Foods implemented**

Since *Escherichia coli* was first identified as a pathogen, many different foods have been suspected or implicated as vehicles in outbreaks of *E. coli* O157:H7, with improperly cooked ground beef being linked epidemiologically to 40% of the disease outbreaks (CDCP, 1996a). From 1982 to 1994, ground beef was associated with 22 outbreaks of *E. coli*

O157:H7 (Doyle *et al.*, 1997). Contaminated beef was responsible for two outbreaks of *E. coli* O157:H7 in 1984, resulting in 48 cases and four deaths (Bean *et al.*, 1990).

Other meat products have also been associated with outbreaks of *E. coli* O157:H7. These include roast beef (Rodrigue *et al.*, 1995), salami (CDCP, 1995), dry-cured salami (CDCP, 1995), fermented salami (CDCP, 1995; Tilden *et al.*, 1996), venison jerky (Keene *et al.*, 1997b) and unrefrigerated sandwiches (Carter *et al.*, 1987). Fruits, vegetables and tubers are not without suspicion since several have been implicated as vehicles of outbreaks. A few examples include potatoes (Morgan *et al.*, 1988), apple cider (Bessere *et al.*, 1993), unpasteurized apple juice (CDCP, 1996b), cantaloupe (Beuchat, 1996; CDPE, 1993; Keene *et al.*, 1993), lettuce (Griffin, 1995b; Mermin *et al.*, 1997), and alfalfa sprouts (CDCP, 1997). Some dairy products, including raw cow's milk (Keene *et al.*, 1997a), yogurt (Morgan *et al.*, 1993) and deer milk (Keene *et al.*, 1997a) have also tested positive for *E. coli* O157:H7. In 1989, an unchlorinated water supply in Missouri was the vehicle of an outbreak resulting in 243 cases and four deaths (Swerdlow *et al.*, 1992). Recreational waters in Oregon (Keene *et al.*, 1994), New York (Ackman *et al.*, 1997) and Illinois (Griffin, 1995b) have also been linked to *E. coli* O157:H7 illnesses.

### **C. Virulence properties**

The term enterohemorrhagic *E. coli* (EHEC) refers to the serotypes of *E. coli* that cause illness similar to that caused by *E. coli* O157:H7. The ability of EHEC strains to attach to and efface with the intestinal mucosal cells, as well as the two cytotoxins it produces, plays a role in the organisms' virulence properties (Karmali, 1989). Enterohemorrhagic *E. coli* produces one or more phage-encoded Shiga-like toxins. The toxins are known as Shiga-like toxin I and II, as well as verotoxins I and II (Karmali, 1989). These toxins are

analogous to Shiga toxins and have a high degree of homology with the Shiga toxins. A large (65-MDa) plasmid, which has been implicated in the bacteria's ability to adhere to intestinal mucosal cells, has been isolated from most EHEC strains isolated from cases involving human illnesses (Toth *et al.*, 1990). Tissue culture assays of the supernatant are used to detect the presence of toxin, or hybridization of the bacterial colony DNA with toxin gene probes are used to characterize Shiga-like toxin producing *E. coli* organisms (STEC) (Levine and Edelman, 1984; Levine *et al.*, 1987).

EHEC is the etiological agent of hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Tarr, 1994). The hemolytic uremic syndrome was first described in 1955 (Gasser *et al.*, 1955). The virulence properties involved in infections caused by *E. coli* O157:H7 are different from those involved with enteropathogenic (EPEC), enterotoxigenic (ETEC) and enteroinvasive (EIEC) *E. coli* (Levine and Edelman, 1984). More than 100 serotypes of STEC have been isolated from animals, food and other sources, but not all of these serotypes have been shown to cause disease in humans (Doyle *et al.*, 1997; Griffin, 1995a). *E. coli* O157:H7 is generally susceptible to the same antimicrobial agents as other Gram negative organisms although antibiotic resistant strains have been isolated (Ratnam *et al.*, 1988). For example, the strain of *E. coli* O157:H7 isolated in the 1990 waterborne outbreak was found to be resistant to streptomycin, sulfamethoxazole and tetracycline (Swerdlow *et al.*, 1990).

#### **D. Symptoms**

Most of the information associated with the illness ascribed to *E. coli* O157:H7 has come from careful epidemiologic field investigations and clinical observations since there is a general lack of effective therapy and the gravity of the infection associated with *E. coli* O157:H7 prohibits volunteer studies from being initiated (Griffin and Tauxe, 1991). It is

believed that there is a three- to four- day incubation period after ingestion of the organism. During this time the bacteria colonize the large intestine and multiply. While most sufferers have non-bloody diarrhea and abdominal cramps, patients that seek medical attention usually have bloody diarrhea. Bloody diarrhea is usually observed during the second or third day of illness (Ostroff *et al.*, 1989). The illness generally lasts for one week, yet 6% of the patients develop the hemolytic-uremic syndrome (HUS) (Griffin, 1998).

#### **E. Infectious doses**

The infectious dose of this organism is believed to be relatively low. An investigation involving the outbreak associated with dry-cured salami estimated the infectious dose to be less than 50 organisms, with some of the cases having an estimated infectious dose of less than five organisms (Tilden *et al.*, 1996). Other investigators estimated the infectious dose of *E. coli* O157:H7 was in the range of two to 45 bacteria in an outbreak involving fermented sausage (Samadpour *et al.*, 1994). Hamburger patties associated with the outbreak in 1993 contained fewer than 700 organisms per patty prior to cooking; therefore, the actual microbial load would likely have been lower at the time of consumption (Griffin, 1995b).

#### **F. Prevalence**

Cattle and other ruminants (Beutin *et al.*, 1993; Kudva *et al.*, 1996) are considered to be major reservoirs for *Escherichia coli* O157:H7, although it has been isolated from other animals such as dogs (Beutin *et al.*, 1993; Treevena *et al.*, 1996), horses (Treevena *et al.*, 1996), swine (Beutin *et al.*, 1993) and cats (Beutin *et al.*, 1993). *E. coli* O157:H7 can also survive in water and cattle feces for many weeks (Wang *et al.*, 1996).

Since cattle are considered to be a major reservoir for the pathogen, USDA-FSIS surveys of cattle herds and meat plants were conducted between 1993-94. The surveys of these meat plants demonstrated that *E. coli* O157:H7 is not routinely present on carcasses or in raw ground beef samples (USDA-FSIS, 1996a; USDA-FSIS, 1996b). No *E. coli* O157:H7 were recovered from the 2,112 cow and beef carcasses sampled between December 1993 and November 1994 (USDA-FSIS, 1996a). Of the 563 raw ground beef samples collected from the meat plants, none tested positive for *E. coli* O157:H7 (USDA-FSIS, 1996b). Although *E. coli* O157:H7 is not routinely present in beef, 25 out of the 63 food borne disease outbreaks (40%) associated with *E. coli* O157:H7 were linked epidemiologically to improperly cooked ground beef (CDCP, 1996a).

Other studies have shown STEC to be present in 15-40% of ground beef samples tested in Canada (Johnson *et al.*, 1996), 23-25% in the U.S. (Acheson *et al.*, 1996; Samadpour *et al.*, 1994), 17% in the United Kingdom (Willshaw *et al.*, 1993) and 16.1% in the Netherlands (Heuvelink *et al.*, 1996). Five of eight veal samples obtained from retail stores in Seattle, Washington tested positive for STEC, as well as 48% of 21 lamb samples tested (Samadpour *et al.*, 1994). Through the use of a shiga toxin gene probe, 18% of pork samples, 12% of chicken, 7% of turkey, 10% of fish and 5% of shellfish samples tested positive for the STEC (Samadpour *et al.*, 1994). In another study, 3.7% (6/164) of beef samples, 1.5% (4/263) of poultry samples, and 2% (4/200) of lamb samples were positive for *E. coli* O157:H7. These samples were obtained from retail stores in Wisconsin and Alberta, Canada (Doyle and Schoeni, 1987). *E. coli* O157:H7 has also been isolated from untreated surface water (McGowan, *et al.*, 1989) and has been shown to survive many weeks in water, especially at low temperatures (Rice *et al.*, 1992; Wang and Doyle, 1996).

## G. Culturing of organism

*Escherichia coli* O157:H7 is a Gram negative, facultative anaerobe bacillus that contains somatic (O) and flagellar (H) antigens (Griffin and Tauxe, 1991). To prepare selective and differential media, certain factors should be considered. Since *E. coli* O157:H7 is an enteric bacterium, selectivity is typically achieved by the addition of bile salts to the media. To distinguish *E. coli* O157:H7 from other *E. coli*, three major culture characteristics are used. These include *E. coli* O157:H7's inability to ferment sorbitol within 24 hours (Doyle, 1984; Farmer and Davis, 1985; Meng *et al.*, 1994; Wells *et al.*, 1983) which is the basis of Sorbitol-MacConkey agar (SMAC). This differential medium has been useful in the isolation of *E. coli* O157:H7 from clinical specimens (March and Ratnam, 1986). Another culture characteristic of *E. coli* O157:H7 is its inability to produce the enzyme Beta-glucuronidase which hydrolyzes 4-methylumbelliferyl-Beta-D-glucuronide (MUG) (Doyle, 1984; Meng *et al.*, 1994; Thompson *et al.*, 1990; Wells *et al.*, 1983). The third culture characteristic of *E. coli* O157:H7 is its poor growth at temperatures above 44°C, a characteristic not shared by other *E. coli* (Meng *et al.*, 1994) bacteria. Two enrichment media often used for culturing *E. coli* O157:H7 are modified *E. coli* broth that contains novobiocin (Okrend *et al.*, 1990) and a double-modified TSB containing acriflavine (Padhye and Doyle, 1991).

Some studies have demonstrated that *E. coli* O157:H7 does not grow well above 41°C; therefore, it may not be detected by standard procedures for enumerating fecal coliforms in food and water (Raghubeer and Matches, 1990). *E. coli* O157:H7 has a generation time of 0.49 hours and 0.6 hours when incubated at 37°C and 42°C, respectively (Doyle, 1984). Padhye and Doyle (1992) reported the organism grew poorly at 44° to 45°C,

with no growth detected at 10°C or 45.5°C following 48 hours of incubation. Others reported that *E. coli* O157:H7 and other serotypes of STEC grew between 10° to 45°C in BHI and *E. coli* broth, with some strains growing at temperatures as low as 8°C (Palumbo *et al.*, 1995). BHI was more supportive than *E. coli* broth at 42°C and 45°C. While *E. coli* O157:H7 grew poorly in trypticase soy broth (TSB) at 44°C and 45°C, it grew best at 37°C in this broth (Doyle and Schoeni, 1984). The variability in growth and survival of *E. coli* O157:H7 at different temperatures may be associated with the media used as well as the specific strains tested.

#### **H. Injured cells**

Sublethally injured cells are characterized by their inability to replicate and form colonies in the presence of selective agents (Ray and Speck, 1973a). Sublethally injured bacteria have a greater demand for nutrient and/or energy requirements compared to fully competent cells (Mackey, 1984; Ray and Speck, 1973a). Freezing injures *E. coli* cells causing them to become more sensitive to bile salts. This enhanced sensitivity prevents the cells from forming colonies on media containing bile salts and other selective agents (Conner and Hall, 1994; Ray and Speck, 1973b). *E. coli* cells injured by a chilling treatment combined with NaCl failed to form colonies on Sorbitol MacConkey Agar. Therefore, SMAC agar may be inadequate for accurately enumerating cells subjected to sublethal stresses (Conner, 1992).

*E. coli* O157:H7 can enter a viable but non-culturable (VBNC) state for several weeks. While in this state it is not detectable by direct plating onto agar plates but its viability can be demonstrated by acridine orange staining (Wang and Doyle, 1996) or by a BacLight molecular probe (Rigsbee *et al.*, 1997).

## I. Heat resistance

Although *E. coli* O157:H7 has been isolated from cooked ground beef patties, studies have demonstrated that it does not possess any unusual heat resistance (Byum *et al.*, 1998; Semanchek and Golden, 1998). Therefore, the meat products incriminated in foodborne disease outbreaks were most likely undercooked. Some D-values for *E. coli* O157:H7 at several different temperatures in ground beef are listed in Table 2. The Z-value of *E. coli* O157:H7 in ground beef was reported at 4.6°C (Line *et al.*, 1991).

**Table 2. D-values for *E. coli* O157:H7 in ground beef.**

Temperature (°C)	Time	Reference
52	78.2-115.5 min	Line <i>et al.</i> , 1991
57.2	270 s	Doyle and Schoeni, 1984
60	45 s	Doyle and Schoeni, 1984
62.8	24 s	Doyle and Schoeni, 1984
64.5	9.6 s	Doyle and Schoeni, 1984

Studies have demonstrated that the internal color of cooked meat is not sufficient to determine the safety of ground beef; therefore, USDA advises consumers to use a meat thermometer when cooking hamburger patties. If the internal temperature of ground beef reaches 160°F (71.2°C), the pathogen should be killed instantly (Doyle and Schoeni, 1984; USDA, 1993). Alternatively, if the ground beef reaches 155°F (68.5°C) and is held at this temperature for 16 seconds, the organism is also eliminated (Doyle and Schoeni, 1984; USDA, 1993). The FDA Food Code requires an internal temperature of 155°F for 15 seconds for cooking ground beef (FDA, 1997).

*E. coli* O157:H7 is more heat resistant in ground beef patties that have been frozen than in patties stored at 15°C. An increase in heat sensitivity is observed if these patties are held at 21°C or 30°C before grilling (Jackson *et al.*, 1996). Beef (ground beef) offers more protection for *E. coli* O157:H7 than chicken, as indicated by a higher population of *E. coli* O157:H7 recovered from ground beef versus chicken. This increase in thermal resistance of *E. coli* O157:H7 in beef compared to poultry products may be attributed to the compositional differences, such as fat content, between the two animal species (Juneja *et al.*, 1997).

Since *E. coli* O157:H7 have been isolated from foods other than ground beef, D- and Z-values have also been determined for different food products. D-values at 50-64°C range from 0.16 to 92.67 minutes for *E. coli* O157:H7 in several other meat products (poultry and pork products) (Ahmed *et al.*, 1995; Doyle, 1984; Line *et al.*, 1991). More than a 5-log reduction in population of *E. coli* O157:H7 in pepperoni was detected at 128°F (53.3°C) after 60 minutes (Hinkens *et al.*, 1996). D-values for *E. coli* O157:H7 isolate 204P in 7% fat meat products were lower than in traditional beef and pork products containing 20% fat (Ahmed *et al.*, 1995). Since apple cider and juice have served as vehicles of this organism, D-values have been derived for these products. At 52°C in apple juice, *E. coli* O157:H7 has a D- and Z-value of 18 min and 4.8°C, respectively. Malic acid found in apple juice may actually sensitize this pathogen to heat if previous acid adaptation has not occurred (Splittstoesser *et al.*, 1996).

## **J. Acid tolerance**

Many STEC strains are able to survive for more than two hours in an acidic environment of a pH less than 2.5 (Benjamin and Datta, 1995; Waterman and Small, 1996). *In vitro* studies have demonstrated that EHEC strains can survive five hours at 37°C in an

acidic environment similar to the human gastric environment (Benjamin and Datta, 1995).

While bacteria can survive and grow in a wide range of pH environments, how the organisms maintain their narrow internal pH range (pH 6.5-8.0) is not clearly understood. Several theories have been proposed. The cytoplasm's buffering capacity is believed to play a large role in intracellular pH homeostasis (Krulwich *et al.*, 1985), yet a low internal pH may induce certain enzymes that may assist in the pH homeostasis (Booth, 1985). Low cell membrane permeability of protons may assist the cell's ability to maintain its internal pH homeostasis as well as the membrane-bound proton pump that acts to extrude protons from the cytoplasm (Benjamin and Datta, 1995). Strong acids will dissociate outside the cell, which prevents the ions from entering the cell. However, if the pH values in the cytoplasm and in the environment are similar, weak acids will enter the cell in their undissociated form and then the proton dissociate once inside the cytoplasm (Salmond *et al.*, 1984).

As bacteria enter stationary phase, specific genes are expressed that may have an effect on the acid tolerance of the bacteria (Martin *et al.*, 1989). Three acid-resistance systems have been identified in the acid tolerant STEC. These include an acid-induced oxidative system, acid-induced arginine-dependent system and a glutamate-dependent system. The oxidative acid-resistance system requires the regulatory gene *rpoS*, but this gene is only partially involved with the arginine- and glutamate-dependent acid-resistance systems (Lin *et al.*, 1996). In a study by Williams and Ingham (1998), a non-pathogenic *E. coli* strain FRIK-124, an *E. coli* O157:H7 43894 strain, and a *rpoS*-deficient mutant of *E. coli* O157:H7 ATCC 43895 were subjected to acid stress (1.5% acetic acid, pH 4.0, 37°C for 15 min). While the acid stress did not significantly enhance subsequent thermotolerance of *E. coli* O157:H7 ATCC 43894, exposing the cells to low temperatures after sequential acid and heat

stresses in TSB enhanced their survival. *E. coli* O157:H7 ATCC 43894 did not decline in population during the acid treatment, yet the acid stress did cause the strain to have significantly lower D-values than non-stressed cells pre-warmed in TSB (54°C). The non-pathogenic *E. coli* strain FRIK-124 did not survive the acid stress, while the *rpoS*-deficient mutant of *E. coli* O157:H7 ATCC 43895 did not decline in number during the acid-treatment and demonstrated no significant change in D-values between the acid-stressed cells versus the non-acid stressed cells. The D-values for the *rpoS*-deficient mutant strain were significantly lower than the D-values for *E. coli* O157:H7 43894 (Williams and Ingham, 1998).

The enhanced survival of *E. coli* O157:H7 under acidic conditions may be attributed to an acid tolerance response (ATR), which is also known as acid habituation. This response refers to an increased resistance to an extreme acid pH shock when cells are cultured in a mildly acidic pH environment (Foster, 1991; Foster and Hall, 1990; Goodson and Rowbury 1989a; Goodson and Rowbury, 1989b). Subjecting microorganisms to sublethal acidic conditions will often cause the organisms to demonstrate an enhanced resistance to severe acid treatment. This phenomenon is known as acid adaptation. Foodborne pathogens such as *Salmonella*, *Listeria* and *E. coli* O157:H7 have demonstrated acid adaptation responses (Foster, 1991; Foster and Hall, 1991; Hill *et al.*, 1995; Garren *et al.*, 1997; Kroll and Patchett, 1992). In some cases the ATR is enhanced at reduced temperatures. In a study where tryptic soy broth and apple juice were acidified with hydrochloric acid or an organic acid before being inoculated with *E. coli* O157:H7, the acid tolerance of *E. coli* O157:H7 was enhanced when stored at 4°C versus 21°C. Acid tolerance was also enhanced when the low storage temperature was combined with a pH 3.5 environment (Przybylski and Witter 1979; Uljas

and Ingham, 1998). Lactic acid (pH 3.5) reduced *E. coli* O157:H7 populations from 5 to 2.4 MPN/ml or  $< 1.5 \log_{10}$  MPN/ml when stored for seven days at 4°C and 21°C, respectively (Uljas and Ingham, 1998).

Some acid-adapted microorganisms have demonstrated the ability of cross-protection to other unrelated stresses (Leyer and Johnson, 1993; O'Driscoll *et al.*, 1996), while some acid-adapted microorganisms become more sensitized to other stresses (Leyer and Johnson, 1997). Inoculated *E. coli* O157:H7 have been observed to survive fermentation, drying and storage of fermented sausage (pH 4.5) for up to two months at 4°C (Glass *et al.*, 1992). Such acid tolerance was attributed to an outbreak involving salami (Tilden *et al.*, 1996). *E. coli* O157:H7 has actually been found in several retail salami samples after more than three months following production (Samadpour *et al.*, 1994). Neither a fermentation step (final pH 4.7) nor heating gradually to 120°F caused a 2-log reduction of *E. coli* O157:H7. However, combining the pH 4.7 environment as achieved by fermentation and the gradual heating to 120°F in 10.5 hours, as used in the manufacturing of Lebanon bologna, a 7-log reduction in *E. coli* O157:H7 population was achieved in the fermented meat product (Ellajosyula *et al.*, 1998).

Cheng and co-workers (2002) demonstrated that acid-adapted *E. coli* O157:H7 ATCC 43889 and ATCC 43895 were more thermal tolerant than their non-adapted counterparts. Acid adaptation of ATCC 43895 resulted in a 10.8-fold increase in the survival percentage, while the acid-adapted cells of ATCC 933 demonstrated only a slightly higher survival percentage over the non-adapted cells (Cheng *et al.*, 2002). When *E. coli* O157:H7 was grown in TSB containing 0-2.5% NaCl, the generation time at 37°C was 0.4-0.5 hours. When the NaCl concentration was increased in TSB, the lag period and generation time also

increased (Glass *et al.*, 1992). Acid-adapted *E. coli* O157:H7 cells are generally less susceptible to NaCl than non-adapted cells. While acid-adapted cells have a greater tolerance for NaCl, both adapted and non-adapted cells show a decrease in survival as incubation time increases. Acid-adapted *E. coli* O157:H7 strains 43889 and 43895 were more thermo tolerant than their nonadapted counterparts, yet acid-adapted strain ATCC 933 demonstrated only a slightly higher survival percentage (1.8%) versus its non acid-adapted counterpart (1.0%). Acid-adapted cells of the three stains tested were not more tolerant to 0.85% bile salts than non acid-adapted cells. After 90 minutes of exposure to 15% ethanol, acid-adapted cells of ATCC 43889 died more rapidly than non-adaptive cells. Similar results were seen with ATCC 43895, yet acid-adapted cells of ATCC 933 showed no significant reduction in survivability throughout the entire exposure time. This finding demonstrates the variation among different strains of *E. coli* O157:H7 (Mackey and Gibson, 1997).

Beef slurries acidified with acetic acid to a pH of 4.70 or 5.00 (common pH values for beef salads) reduced *E. coli* O157:H7 populations, yet in beef slurries acidified to pH 4.70 or 5.40 with citric and lactic acids, populations did not decline during 24 hours of incubation at 30°C. Ground roast beef salads containing 40% mayonnaise (pH 5.94-6.07) and held at 5°C for up to 72 hours did not cause any changes in the population of *E. coli* O157:H7 (Abdul-Raouf *et al.*, 1993).

Bacterial attachment to lean beef surfaces is partly due to a net negative charge on bacterial cell walls (Dickson and Koohmaraie, 1989). The ability to remove these cells depends on the stage of attachment between the bacterial cells and beef surfaces (Firstenberg-Eden, 1981). While acid spray washing will reduce the total microbial populations on beef carcass surfaces, it will not completely eliminate acid-tolerant *E. coli*

O157:H7 (Brackett *et al.*, 1994; Cutter and Siragusa, 1994). Three popular treatments to reduce microbial levels on carcasses have been investigated. These include the use of: 1) acetic acid, lactic acid and trisodium phosphate (Dorsa *et al.*, 1997b), 2) steam vacuuming, and 3) hot water spray washing (Dorsa *et al.*, 1997a). While there was a reduction in the number of *E. coli* O157:H7, the pathogen was not eliminated from the beef carcasses. This lack of complete inactivation of *E. coli* O157:H7 by organic acid carcass washes and hot or warm water sprays also demonstrates the organism's tolerance for acidic conditions (Cutter and Siragusa, 1994).

#### **K. Freezing**

There appears to be conflicting data as to the effect of cold storage and freezing on the survival of *E. coli* O157:H7. In one study, 114-g beef patties were inoculated with the pathogen and then stored at -18°C for eight days or at 3°C for nine hours. The organisms recovered from the frozen patties proved to be more heat resistant than those stored under refrigeration temperatures ( $p < 0.05$ ) (Jackson *et al.*, 1996). In a second study, *E. coli* O157:H7 cells inoculated in ground beef (3g) and stored at refrigeration temperatures (4°C, 48h) were more heat resistant to 60°C than those inoculated and stored at -18°C for 48 hours (Juneja *et al.*, 1997). The reasons for the contrasting results could be due to the different sized samples used and therefore different heating times, to the induction of cold-shock proteins, or to biochemical changes in bacterial membranes resulting from storage at low temperatures for different time periods. Other evidence suggests that *E. coli* O157:H7 can survive for up to nine months in frozen ground beef with no major population change if the product is initially stored at -80°C and then held at -20°C (Doyle and Schoeni, *et al.*, 1984).

#### **IV. Emulsified Poultry Products**

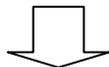
Over the past thirty years the consumption of chicken has more than doubled in the U.S., with the per capita consumption of chicken being approximately 54 lb per person in 1999. This increase is due in part to health-related concerns and new convenient poultry food products that are further processed (USDA-ERS, 2000). The poultry industry began marketing more cut-up and further processed products in the 1950's and 60's, which increased the use of meat mechanically separated from parts such as frames, necks, backs, etc. The mechanically separated meat is used in emulsified products such as bologna, salami, frankfurters, etc. (Froning and McKee, 2001). Before emulsified products are produced, the meat ingredients are tested using AOAC methods to determine the moisture, fat and protein content. The meat pre-blends and lean meats are combined with salts, alkaline phosphates, curing ingredients such as nitrite and erythorbate, half of the required water (iced), and then mixed in a vacuumed bowl chopper. The mixture is emulsified to a paste while maintaining a temperature of less than 40°F. If powdered CO<sub>2</sub> is used, it is added at this point and the mixture is allowed to rest for approximately five minutes to extract proteins. Other fat trimmings are added in addition to seasonings, spices, additives and the rest of the ice water slush. This mixture is homogenized in the bowl vacuum under a 60% vacuum. The mixture is maintained at a temperature less than 50°F. If a smaller particle size or a smoother texture is needed, the mixture is then passed through an emulsion mill with the final temperature being approximately 55°F. At this point the mixture is vacuum stuffed into a moisture proof fibrous casing for bologna and a cellulose casing for frankfurters. These products are fully cooked using a multiple-stage cooking cycle. If the mixture contains  $\leq 100$  ppm of nitrite, the internal temperature must reach 71.1°C or 68.3°C if the mixture contains  $>100$  ppm nitrite.

After the product is thermally processed it is chilled to  $\leq 40^{\circ}\text{F}$  and held in a tempering cooler at  $26\text{-}28^{\circ}\text{F}$  before being sliced (bologna) or peeled (frankfurters) (Keeton, 2001). All pathogenic bacteria present in the raw product are eliminated during the cooking process. It is during the slicing and peeling stage that the ready-to-eat product can be recontaminated with pathogens present in the processing environment (Keeton, 2001). Some research studies have shown that the equipment used for slicing, dicing, packaging and brining harbor pathogens, namely *L. monocytogenes* (Autio *et al.*, 1999; Miettinen *et al.*, 1999; Tompkin *et al.*, 1999). After the product is sliced or peeled, the product is then packaged and placed in cold storage to await shipping (Keeton, 2001). A flow chart of this process is summarized in Figure 1.

**Figure 1. Poultry Frankfurter/Bologna Processing Procedures (adapted from Keeton, 2001).**

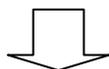
### RAW MATERIALS

Mechanically separated poultry meat, select fresh/tempered chilled boneless breasts, thighs, drumstick meat, trimmings or pre-blends (26-30°C)



### ANALYSIS OF MEAT

Using AOAC tests analyze meats for moisture, fat and protein



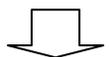
### FORMULATION

Using least cost formula to reach compositional endpoint constraints (15% or 0.5% fat)



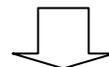
### GRINDING

Grind lean meat through 0.125" plate. Not required for mechanically separated poultry.



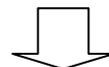
### CHOPPING/HOMOGENIZATION

1. Combine pre-blend or lean meats with salt, alkaline phosphates and cure ingredients such as nitrite and erythorbate with ½ water as ice slush into a vacuum bowl (80% vac).
2. Emulsify to a paste consistency, maintaining a temperature of ≤40°F. If needed, add powdered CO<sub>2</sub>. Rest the meat blend for ~5 minutes to extract protein.
3. Combine fat trimmings, additives, seasons, and spices with remaining ice water slush. Homogenize (60% vac) to paste consistency, while maintaining a temperature of ≤ 50°F.



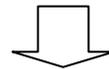
### EMULSIFICATION

To ensure a smooth emulsion, pass mixture through an emulsion mill, with end temperature reaching approximately 55°F.



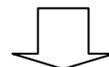
### FOAMING

Vacuum stuff mixture into a moisture-proof fibrous casing for bologna or a peelable collagen casing for frankfurters.



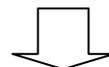
### THERMAL PROCESSING

Using a stage cook cycle, cook until the internal temperature reaches 71.1°C if product contains <100 ppm of nitrite or to 63.3°C if product contains >100 ppm nitrite.



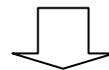
### CHILLING

Cool product on cooler racks to ≤40°F, in a blast chiller (-10°F), and then hold in a tempering cooler (26-28°F) before slicing/peeling.



### PEELING/SLICING/PACKAGING

Slice product (bologna) or remove casing (frankfurters).



### PRE-SHIPING STORAGE

After boxing, hold product at 32°F or <0°F

## **V. Packaging**

Barron (1995) explained there are four main functions of food packaging: containment, a means to display information about the product, convenience and protection of the food product. Protection is the most important of the four functions. With proper packaging the product can be protected from environmental contaminants such as dust, rodents and microorganisms. The product can also be protected from humidity, light and oxygen.

Typically, there are three categories of packaging: primary, secondary, and tertiary. Primary is the packaging material which comes in contact with the food product and what consumers see when the food is purchased at the retail level. Materials used for primary packaging can be fiber-based (paper, paperboard), glass, metal and plastics, or a combination of multiple types of materials. Plastics (polymers) are the most common material used with poultry products, be it a plastic coating, lining, overwrap or bag. This is due to the versatility, cost and convenience of the different polymers available. Considerable research is underway to develop primary packaging materials that increase product shelf-life and reduce microbial loads in products. These advances will be discussed in greater detail later. Labeling containing product information deemed necessary by the food processor and government will be found at this level of packaging. Next is the secondary packaging. This level of packaging may be a case, an outer box or wrapper that holds several primary packages together. The secondary packaging will not only ease the handling of primary packages but will also protect the products from damage they may receive during shipping and handling. Secondary packages are stacked to form pallet size units. These pallet units

are held together with tertiary packaging such as a stretch wrap. This final level of packaging eases the shipping and handling of products (Dawson, 2001).

In order to meet consumers' ever increasing demands for "fresh-like" products, advances in food technology, biotechnology, packaging and material sciences have been combined (Miltz *et al.*, 1997) in an effort to form "active" packaging materials. This type of packaging provides more than an inert barrier between the product and the external environment (Hotchkiss, 1995). One example of active packaging is incorporating antimicrobial agents directly into polymeric packaging. This innovation would allow the food industry to combine the merits of existing packaging with the preservative functions of antimicrobials. Examples of active packing include, but are not limited to. incorporation of silver-containing zeolite to contact surfaces of polymer films (Nielsen, 1997) and incorporating imazalil into low-density polyethylene for fruit and vegetable wrapping (Hale *et al.*, 1986) and cheeses (Weng and Hotchkiss, 1992). However, to achieve regulatory approval for such product applications requires extensive work. Often it is easier to obtain government approval with natural occurring antimicrobials such as nisin. This approach may be defined as "biopreservation" of foods that involves inhibiting or destroying unwanted microorganisms in foods through the use of antagonistic microorganisms (Stiles, 1994) or metabolites of microorganisms (Motlagh *et al.*, 1991).

In order to improve the safety and quality of processed foods, the use of antimicrobial edible films and coatings have been a point of keen interest for some time (El Ghaouth *et al.*, 1991; Fang *et al.*, 1994; Kesler and Fennema, 1986, 1989a, 1989b; Muzzarelli *et al.*, 1990; Natrajan and Sheldon, 2000a and 2000b; Rico-Pena and Torres 1990; Siragusa *et al.*, 1999). Several investigations have involved incorporating nisin into an edible hydroxy propyl

methyl cellulose (HPMC) film or coating. Since the cellulose based films are hydrophilic in nature, stearic acid was added to improve the moisture barrier properties. As the quantity of stearic acid was increased in the HPMC mixture, the anti-microbial effect of nisin against *Listeria innocua* was shown to decrease (Coma *et al.*, 2001). However, a significant difference in the degree of inhibition of *L. innocua* was detected between a nisin-free coating and a nisin-containing coating without stearic acid. It was theorized that this inhibition of *L. innocua* might have been due in part to the limitation of gas transfer through the HPMC coating and not wholly due to the presence of nisin. When *S. aureus* was tested in this system, higher concentrations of nisin resulted in more inhibition of *S. aureus*. With the exception of films without stearic acid, a significant improvement in inhibition was seen with higher concentrations of nisin at both microbial loads. Cellulosic coatings with  $5 \times 10^3$  IU per ml of pure nisin and no stearic acid prevented *L. innocua* and *S. aureus* surface growth. Since stearic acid was used to improve the water vapor barrier properties, yet decreased the efficacy of nisin, the actual usefulness of these films and coatings is questionable (Sebti and Coma 2002).

The efficacy of bacteriocins to act as food preservatives is often inconsistent due to uneven distribution within the food system. Therefore, a reliable delivery system is needed to maximize the biopreservative potential of the antimicrobials (McMullen and Stiles, 1996). Sprinkling bacteriocin preparations directly onto barrier bags (Miltz *et al.*, 1997) and immobilization onto edible films (Cutter and Siragusa, 1997) have shown some promise in successfully delivering bacteriocins to the surface of foods. Not only should the bacteriocin have a low diffusibility in the host film but it should also remain on the surface of the food

since diffusion into the food would result in a reduction in concentration thus allowing surface microorganisms to overcome the bacteriostatic effects (Greer, 1981).

## **VI. Bacteriocins of Gram Positive Bacteria**

The term “bacteriocins” was first proposed by Jacob in 1953. It includes all entities (i.e. proteinaceous molecules) with a bactericidal activity against other closely related bacteria (DeVuyst, 1994; Jacob *et al.*, 1953). Bacteriocins are biologically active proteins or protein complexes such as protein aggregates, lipocarbohydrate proteins, and glycoproteins (DeVuyst and Vandamme 1994b). This heterogeneous group of antibacterial proteins varies in mode of inhibitory action, spectrum of activity, molecular weight, genetic origin and biochemical activity (Abee *et al.*, 1995). Under most treatment conditions bacteriocins are more bactericidal than bacteriostatic. While the target site of most bacteriocins is the cytoplasmic membrane, some bacteriocins can also damage RNA or DNA of sensitive cells (Hurst and Hoover, 1993; Ray, 1992). Bacteriocins produced by Gram positive bacteria have a broader spectrum of inhibitory activity against other Gram positive organisms as compared to the colicins produced by Gram negative bacteria. Bacteriocins vary in molecular weight from 100 Da to 40,000 Da (Tagg *et al.*, 1976). Cell receptors are not always necessary for their action. Genetic determinants can be plasmid-mediated as well as chromosomally encoded (Tagg *et al.*, 1976).

Because bacteriocins are proteinaceous substances they are generally inactivated by an array of proteolytic enzymes including trypsin, alpha-chymotrypsin, pepsin and Proteinase K. Since bacteriocins are sensitive to proteolytic enzymes of gastric and pancreatic origins, bacteriocins used in foods as biological preservatives presumably will not affect the microflora of the gastrointestinal tract. Bacteriocins produced by different bacteria generally

vary in sensitivity to these proteolytic enzymes (DeVuyst 1994). For example, low molecular weight bacteriocins are more susceptible to trypsin digestion but are less likely to be inactivated by heat when compared to high molecular weight bacteriocins (Tagg *et al.*, 1976).

It is very promising that bacteriocins produced by lactic acid bacteria can be successfully used as biological food preservatives (DeVuyst, 1994) because of their inhibitory properties (Bender and Brodsky, 1990). This assumption is key since the tendency of today's culture is to decrease the use of chemical additives in foods by replacing them with more natural products. Bacteriocins could potentially replace the use of such chemical preservatives as sulfur dioxide, benzoic acid, sorbic acid, nitrates and nitrites (Lloyd and Drake, 1975). Many bacteriocins are heat stable, therefore they can be used in applications involving a heat treatment. It appears bacteriocins have a universal bactericidal and irreversible mode of action. Several bacteriocins are known to be active against food-spoilage and food borne pathogens such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes* and *Staphylococcus aureus* (DeVuyst, 1994).

There are four major classes of bacteriocins produced by lactic acid bacteria: (I) Lantibiotics, (II) small heat stable proteins, (III) large heat liable proteins and (IV) complex proteins whose activity requires association of carbohydrates or lipid moieties (Klaenhammer, 1993). Lantibiotics include nisin and three other bacteriocins that have molecule structures similar to nisin. These are produced by *Lactococcus lactis* (Lactincin 481), *Lactobacillus sake* (lactocin S) and *Carnobacterium piscicola* (carnocin UI49) (Klaenhammer, 1993; Mortvedt-Abildgaard *et al.*, 1991; Piard *et al.*, 1992; Stoffels *et al.*,

1992). Lantibiotics are membrane active peptides that contain the thioether amino acids lanthionine and beta-methyl-lanthionine in addition to other modified amino acids such as dehydrated serine and threonine (Jung, 1991a). While peptide antibiotics are synthesized from amino acid complexes via several enzyme systems, nisin and other lantibiotics are synthesized by post-translational modification of a gene-encoded protein (Thomas *et al.*, 2000). The synthesis of nisin will be discussed in more detail in the next section.

## **A. Nisin**

### 1. History-Discovery

The production of an antagonistic substance other than the metabolic end products of lactic acid bacteria was reported in 1928 for an “inhibitory” streptococci during the slow acid production in cheese making. The antagonistic activity of *Lactococcus lactis* subsp. *lactis* (formerly known as *Streptococcus lactis*) was against *Lactobacillus delbrueckii* subsp. *bulgaricus* (Roger, 1928). It was later confirmed that this substance was a polypeptide (Whitehead, 1933). Since *L. lactis* subsp. *lactis* belongs to the Lancefield serological group N, the name “nisin” was derived from “group N inhibitory substance” (Mattick and Hirsch, 1947). The “-in” suffix was used because this substance was first categorized as an antibiotic (Mattick and Hirsch, 1944). Since the term antibiotic defines a culture-induced antagonism which cannot be attributed to organic acids, hydrogen peroxide or bacteriocins, nisin is not an antibiotic (Klaenhammer, 1988). It is now known that nisin does not persist in the body or environment. Therefore, no bacterial resistance to nisin or to any medically important antibiotic has occurred through the use of nisin. This is a key attribute that makes most antibiotics unsuitable for use in foods (Thomas *et al.*, 2000). Because nisin is degraded by intestinal proteases and peptides (Fraser *et al.*, 1962), there have been no reports of adverse

side effects from the consumption of nisin in humans and relatively low toxicity seen in rats (Delves-Broughton, 1990; Frazer *et al.*, 1962; Hurst, 1981; Lipinska, 1977).

The first commercial preparation of nisin, Nisaplin, was developed by Aplin and Barrett (Dorset, England) between 1962 and 1965. Its sole purpose was to be used within the company to prevent clostridial spoilage of processed cheese. Because this was a common problem in the cheese industry, Nisaplin was soon being used by other cheese processors in the UK as well as in other countries (FAO/WHO, 1969). Nisaplin contains 2.5% nisin, 74.7% sodium chloride, 1.7% moisture, 5.93% carbohydrates, 17.12% protein and traces of fat (Eapen *et al.*, 1983).

Nisin was also tested during World War II not only to solve spoilage problems associated with cheese making, but also in human or veterinary medicine which proved to be unsuccessful at the time (Mattick and Hirsch, 1944; Meanwell, 1943).

## 2. Laws and regulations

It was suggested that nisin be used as a food preservative in 1951 (Hirsch *et al.*, 1951). By 1969, nisin was recognized as a safe and legal biological food preservative by a joint FAO/WHO expert committee on food additives (FAO/WHO, 1969). Since then, nisin has received GRAS (generally recognized as safe) status in the United States (Federal Register, 1988) and was originally approved for use in some pasteurized cheese spreads to prevent spore outgrowth and toxin production by *Clostridium botulinum* (Delves-Broughton, 1990). Nisin has also been approved for use as a food preservative in more than 40 other countries. It can be used as a direct food additive for extending shelf life of dairy products or produced in foods as a byproduct of a bacterial fermentation. Nisin can also be used in canned foods for the prevention of spoilage caused by thermophilic microorganisms (Fowler

and McCann, 1971; Fowler and McCann, 1972). While nisin can prevent spoilage of canned foods, it is ineffective in preventing spoilage if poor-quality starting materials are used (Gibbs and Hurst, 1964). Moreover, the inhibitory effectiveness of nisin against sensitive bacteria decreases as microbial loads increase (Scott and Taylor, 1981).

### 3. Production of nisin

Nisin is produced during the exponential growth phase of *L. lactis* subsp. *lactis*, with production ceasing once the cells enter stationary phase (Buchman *et al.*, 1988; Hurst, 1966; Hurst and Kruse, 1972). If the lag growth phase is extended, the amount of nisin produced will increase (Hurst and Dring, 1968). The pH of the propagation medium plays a key role in the release of nisin from the cells (Hurst and Dring, 1968; Hurst and Kruse, 1972; White and Hurst, 1968). If the pH of the environment is  $<6.0$ , more than 80% of the nisin will be released from the cell. In contrast, if the pH of the media is  $>6.0$ , most of the nisin will remain in the cell.

Nisin, like all lantibiotics, is synthesized on the ribosome as a precursor or pre-peptide that is enzymatically modified following translation (de Vos *et al.*, 1995). It is suggested that the synthesis and export of lantibiotics from the cell occurs in three phases. An intracellular post-translational modification, in which several amino acids residues are modified, comprises the first phase. During this phase some hydroxyl amino acids are dehydrated to form dehydroamino acids, as well as thioethers are formed by the addition of a sulfhydryl group. The modified peptide is exported across the cytoplasmic membrane during the second phase. In phase three, the leader peptide is cleaved proteolytically, producing the final molecule (Schnell *et al.*, 1988).

#### 4. Structure

Lanthionine rings with monosulfide bridges are characteristic of the lantibiotics. Since nisin is composed of a linear structure, it is classified into the Group A as opposed to Group B lantibiotics that are circular in structure (Jung and Sahl, 1991). Lantibiotics in Group A are cationic and amphiphilic peptides with elongated forms. Because nisin has a high proportion of basic amino acids, the molecule has a net positive charge (Gross and Morell, 1971). Gross and Morrell (1971) were the first to propose the complete primary structure of nisin as seen in Figure 2. This structure was later confirmed through chemical syntheses by Furkase *et al.* (1988) and NMR spectroscopy (Chan *et al.*, 1989). It is a 34 amino acid polypeptide described as a heterodetic pentacyclic subtype A lantibiotic. A mature nisin peptide has dehydrated residues of dehydroalanine and dehydrobutyrine that are derived from serine and threonine residues, respectively, as well as lanthionine in Beta-lanthionine residues that form five intramolecular thioether bridges (Jung and Sahl, 1991). While the monomer of nisin has a molecular mass of 3,353 Daltons (Gross and Morell, 1971; Jung, 1991a; Jung, 1991b), it usually occurs in the more stable dimer form (7 kDa). It can also occur as a tetramer (14-kDa) (Cheeseman and Berridge, 1959; Ingram *et al.*, 1967; Jarvis *et al.*, 1968).

Nisin A is the most studied member of the lantibiotics. It is produced by several strains of *Lactococcus lactis*, including *L. lactis* subsp. *lactis* NCFB 497 (Hirsch *et al.*, 1951). Studies have shown that it has a wide range of inhibitory effects against Gram positive bacteria (Hurst, 1981; Jung and Sahl, 1991). Nisin Z is a naturally occurring variant of Nisin A. It is produced by *L. lactis* subsp. *lactis* NIZO 22186, Strain N8, Strain SIK-83 and others (Graeffe *et al.*, 1991; Kuipers *et al.*, 1991; Mulders *et al.*, 1991). The structural difference

between the two is the substitution of histidine for asparagine at position 27 in Nisin Z (Mulders *et al.*, 1991).

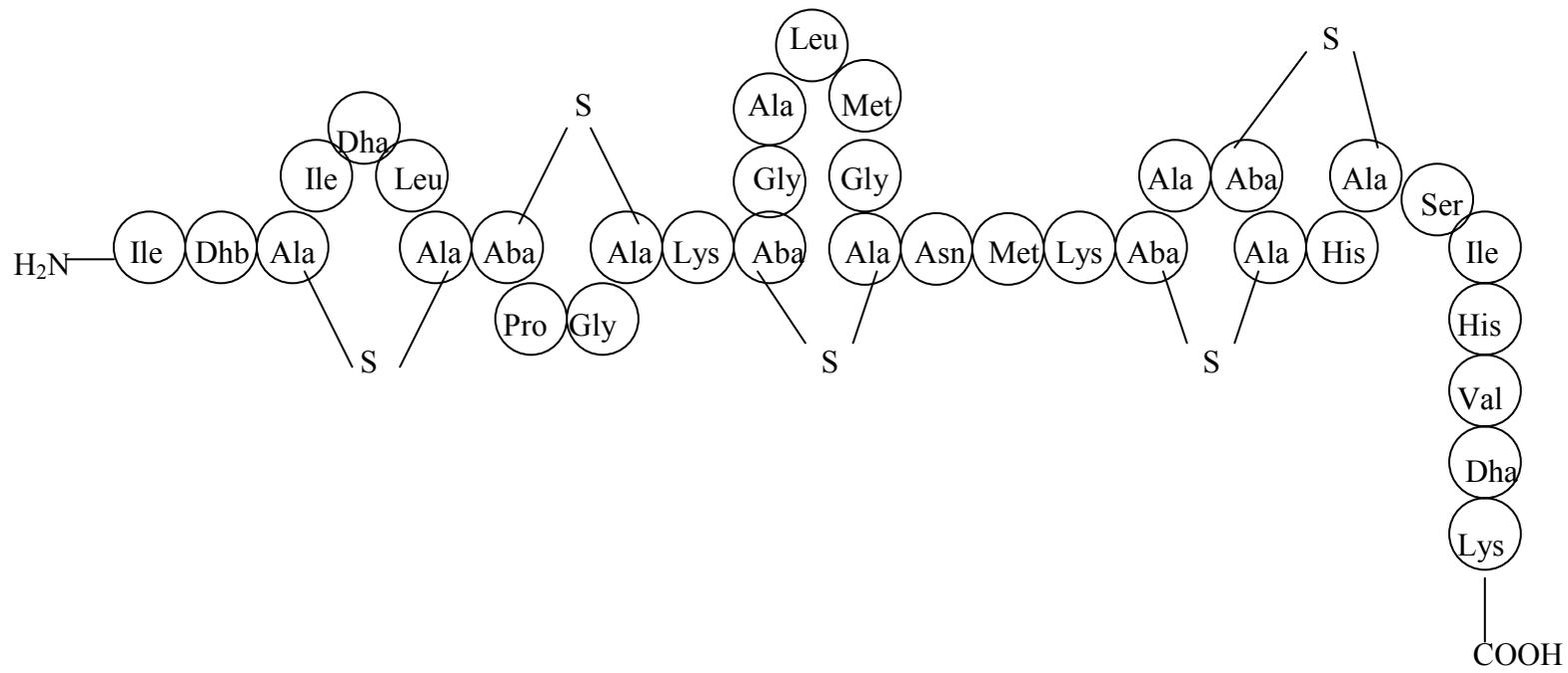


Figure 2. Structure of Nisin.

## 5. Storage

Bacteriocin preparations have often been shown to be less stable with increased purification (Tagg, *et al.*, 1976), yet peptides are stable for several months when refrigerated, frozen or freeze dried and remain biologically active under acidic conditions (Ray, 1992). If stored in a dark, dry environment, with temperatures not exceeding 25°C, nisin concentrates will remain stable for extended amounts of time (Tramer 1964; 1966). Nisin is an acidic molecule that is more stable and easier to solubilize at lower acidic pHs (Hurst, 1981; Thomas and Wimpenny, 1996).

## 6. Mechanism of Inhibitory Action

### *i.* Spores

There are three development stages for spore germination. These include germination swelling, pre-emergence swelling, and emergence and elongation (Gould, 1964). The stage of development of spore germination may dictate if nisin has a sporicidal or sporostatic effect on spores.

Some believe nisin's activity against spores is more bacteriostatic than bacteriocidal (Delves-Broughton and Gasson, 1994; Montville *et al.*, 1995). Nisin's inhibitory effect against bacterial spores can occur after spore germination, preventing post-germination swelling and subsequent spore outgrowth (Attwell *et al.*, 1972; Gould, 1964; Hitchens *et al.*, 1963). Molecular studies suggest this is caused by the binding of some dehydroalanine groups contained in nisin to the sulfhydryl groups found in the membranes of newly germinated spores that are believed to be the target of nisin activity (Morris *et al.*, 1984). This prevents the outgrowth of the spore, thus inhibiting the formation of the vegetative cell. It is believed that this sporicidal activity of nisin is usually confined to clostridial spores (Ramseir, 1960).

Hirsh and Grinstend (1954) demonstrated this inhibitory activity of nisin against clostridial spores to be inversely related to the concentration of spores. If nisin has a sporastatic effect on spores, the spore will undergo germination swelling and nisin will prevent the pre-emergence swelling which precedes the emergence of the vegetative cell (Gould and Hurst, 1962). Generally spores are more sensitive to nisin than are vegetative cells. Sensitivity of spores to nisin is increased if the spores are injured (*i.e.* through heat or other treatments), and are suspended in an acidic environment (Thomas *et al.*, 2000).

## ii. Gram Positive Bacteria

Inhibition of Gram positive microorganisms by nisin is attributed to the protein's ability to be absorbed by vegetative cell membranes leading to a disruption of the membrane through membrane insertion, pore formation and simultaneous depolarization. By increasing the membrane permeability the membrane transport is disrupted inhibiting energy production and biosynthesis of proteins and nucleic acids. Nisin is also able to induce autolysis of sensitive bacterial cells (DeVuyst and VanDamme, 1994a). Nisin's lethal effect is greatest against cells that are actively growing because the membranes are fully energized (Maisnier-Patin and Richard, 1996; Sahl, 1991). When pore formation occurs, leakage of small molecules including  $K^+$  ions, solutes and metabolites such as ATP and amino acids occurs. This depletes the proton motive force of the cell that starves the cell of energy and ends certain biosynthetic processes (Ruhr and Sahl, 1985).

Nisin also inhibits the growth of Gram positive bacteria by interaction with anionic constituents of the negatively charged cell wall. This results in a rapid and specific efflux of cytoplasmic cell constituents leading to an interference of the energy metabolism of the cell (Bierbaum and Sahl, 1985; Ruhr and Sahl, 1985). This peptide also acts on energized

membrane vesicles to disrupt the proton motive force, inhibiting the uptake of amino acids and causing a release of accumulated amino acids (Jung and Sahl, 1991). The primary target of nisin is the cytoplasmic membrane. It has been shown that nisin will associate with non-energized liposomes, the greatest interaction being with negatively charged phospholipids. This interaction indicates that the initial association is at least in part charge dependent (Abee *et al.*, 1994; Gao *et al.*, 1991; Garcia-Garcera *et al.*, 1993; Kordel *et al.*, 1989). Studies have demonstrated that the dehydroalanine and dehydrobutyrine residues in active nisin play a role in the inhibitory sequence by acting as electrophilic Michael acceptors toward nucleophiles in the cytoplasmic membrane (Liu and Hansen, 1990).

Nisin has a relatively broad spectrum of antimicrobial activity against Gram positive bacteria and bacterial spores. Some of these microorganisms include *Lactococcus lactis* subsp. *cremoris*, staphylococci, clostridial species and *Listeria monocytogenes* (Hurst, 1978; Hurst, 1981; Klaenhammer, 1988; Lipinska 1977; Mattick and Hirsh, 1944). The inhibitory effect of nisin can be either bactericidal or bacteriostatic. This response is dependent on the overall environmental conditions of the treated product (Delves-Broughton *et al.*, 1996). Sensitivity to nisin varies greatly among strains and even in the same species (Ferreira and Lund, 1996; Gupta and Prasad, 1989; Meghrous *et al.*, 1999; Ramseier, 1960; Ukuku and Shelef, 1996). The effectiveness of nisin decreases with an increase in population of the target organism (Rayman *et al.*, 1981; Scott and Taylor, 1981).

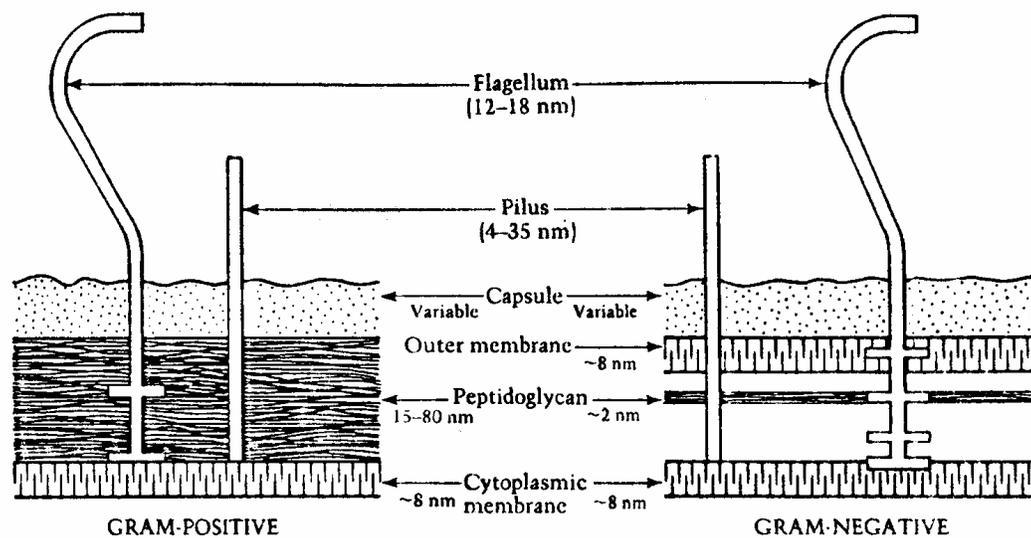
### *iii.* Gram Negative Bacteria

The structure of the cell wall is different between Gram negative and Gram positive bacteria (Figure 3), and therefore each is affected differently by antibiotics, detergents and bacteriocins. The outer membrane of Gram negative bacteria is composed of phospholipids,

proteins and lipopolysaccharides (LPS). This membrane acts as a permeability barrier for the cells, preventing molecules such as antibiotics, detergents and dyes from reaching the inner cytoplasmic membrane (Nikaido and Vaara, 1987). While the outer membrane is impermeable to most molecules, porins are present that will allow molecules with molecular weights of less than 600 Da to diffuse through freely. The smallest bacteriocins produced by lactic acid bacteria are approximately 3 kDa. Therefore, the size of the bacteriocins prevents it from reaching the cytoplasmic membrane of Gram negative bacteria, which is the target site of nisin activity (Klaenhammer, 1993; Kordel *et al.*, 1989; Stiles and Hastings, 1991). Because of this, Gram negative bacteria are generally not sensitive to nisin (Cutter and Siragusa, 1995a; Hurst, 1981; Spelhaug and Harlauder, 1989; Stevens *et al.*, 1991; Stevens *et al.*, 1992;).

Studies have shown that some Gram negative bacteria are sensitized to nisin after being exposed to treatments that change the permeability of their outer membrane (Ray, 1993; Stevens *et al.*, 1991). Alteration of the outer membrane of *E. coli* caused by physical treatments, such as osmotic shock, increases the organism's sensitivity to nisin (Kordel and Sahl, 1986). Sublethally acid-injured Gram negative bacteria *Yersinia enterocolitica* and *Pseudomonas fluorescens* were sensitive to nisin during *in vitro* studies (Kalchayanand *et al.*, 1992). Other studies have shown that employing high concentrations of nisin ( $\geq 470\mu\text{M}$ ) alone can totally inhibit *E. coli* O157:H7 (Alexandra *et al.*, 1996). However, by adding chelators (Cutter and Siragusa, 1995a; Fang and Chen, 1997) or pre-treating cells with ultra high pressure (Kalchayanand *et al.*, 1992; Steeg *et al.*, 1999), nisin's inhibitory activity against *E. coli* O157:H7 was enhanced.

Figure 3. Comparison of cell wall structure of Gram positive and Gram negative bacteria (Ingram *et al.*, 1983).



## B. Chelating agents

Although Gram negative bacteria are not generally as sensitive to nisin as are Gram positive bacteria, by combining chelating agents with nisin, activity of nisin can be extended to Gram negative bacteria (Blackburn *et al.*, 1989; Stevens *et al.*, 1991). The lipopolysaccharide (LPS) layer of the outer membrane of Gram negative bacteria contains magnesium and calcium ions that help stabilize the LPS layer and prevent the penetration of molecules such as antibiotics, detergents, dyes and nisin (Leive, 1974; Nikaido and Vaara, 1987; Ray, 1993; Stevens *et al.*, 1991). Studies have shown that while Gram negative bacteria are normally insensitive to such antibiotics as actinomycin and rifampicin, by exposing the cells to EDTA their susceptibility increased (Leive, 1974; Vaara and Nikaido, 1984). Other studies have demonstrated when nisin is combined with food grade chelating agents such as EDTA, citric acid, etc., the chelators alter the outer membrane of Gram negative bacteria resulting in an increase in cellular sensitivity. This perturbation of the outer bacterial membrane allows nisin access to the cytoplasmic membrane of Gram negative organisms (Blackburn *et al.*, 1989; Delves-Broughton, 1990; Hancock, 1984; Stevens *et al.*, 1991). This effect is due to the chelation of divalent (*i.e.*  $Mg^{2+}$ ,  $Ca^{2+}$ ) cations located in the LPS layer of the outer membrane, causing an increase in outer membrane permeability (Stevens *et al.*, 1991).

Immediately following treatment with EDTA, 30-50% of the lipopolysaccharides and smaller amounts of other lipids and proteins are released from the outer membrane of Gram negative bacteria (Leive 1974; Nikaido and Vaara, 1985 and 1987). Another study has demonstrated that EDTA will increase the permeability of the outer cell membrane of *E. coli*

and *S. typhimurium* without altering the cytoplasmic membrane. This treatment increased cellular sensitivity to nisin but not to pediocin SJ-1 (Chikindas *et al.*, 1993).

In a study by Stevens *et al.*, (1992), ten different Gram negative organisms were cultured to a population density of  $5 \times 10^7$  cfu/ml and used to determine the efficacy of four different chelators to increase their sensitivity to nisin. The chelators were EDTA, ethyleneglycol tetra acetic acid (EGTA), citrate acid monohydrate (citrate) and sodium phosphate dibasic (phosphate). Cells were concentrated by centrifugation and resuspended in a cell buffer containing 50  $\mu$ g/ml nisin and 20mM of each chelator. The pH of the suspensions was 6.5 except for the phosphate/nisin treatment which was 7.0. After incubating the cell suspensions for 60 min at 37°C, the mean log reduction across all organisms was 5.9, 3.8, 5.1 and 4.0 for EDTA, EGTA, citrate and phosphate, respectively. The reduced inhibition of the phosphate treatment may be due to a decrease in nisin activity as the pH increased (Hurst, 1981; Liu and Hansen, 1990). In comparison to EDTA, EGTA exerted significantly less effect on Gram negative organisms when combined with nisin (Stevens *et al.*, 1992). While EDTA strongly binds to a variety of divalent cations, including calcium and magnesium, EGTA only binds to calcium ions. Thus, EGTA should be less effective than EDTA in sensitizing Gram negative bacteria to nisin (Nikaido and Vaara, 1985; Stevens *et al.*, 1992).

A wide variety of *Salmonella* serotypes and other Gram negative bacteria are inactivated when exposed to a combination of EDTA and nisin (Blackburn *et al.*, 1989; Stevens *et al.*, 1991). Optimum nisin inactivation of Gram negative bacteria is seen with EDTA concentrations ranging from 0.1-20 mM (Blackburn *et al.*, 1989). Several serotypes of *Salmonella* as well as several *E. coli* strains (including *E. coli* O157:H7 HC 19386) were

highly sensitive to a combination of 20 mM EDTA and 50 µg/ml nisin when suspended in a cell buffer (Stevens *et al.*, 1991). Generally, greater inactivations of *Salmonella* serotypes were observed when exposure temperatures were increased (4°C, 20°C, 30°C, 37°C and 42°C) during exposure to 50 µg/ml of nisin and 20mM EDTA (Stevens *et al.*, 1992). While there is generally a significant decrease in cell population when suspended in a solution, nisin (50 µg/ml) combined with 50 mM EDTA yield no more than a log 0.42 reduction in *E. coli* O157:H7 population per cm<sup>2</sup> of beef stored for three days at 4°C (Cutter, 1995a). Similarly, Zhang and Mustapha (1999) compared a nisin or nisin/EDTA combination with an untreated control, and observed no statistically significant treatment differences in *E. coli* O157:H7 populations on beef samples although cells treated with nisin had numerically lower populations.

### **C. Environment**

The sensitivity of microorganisms to the inhibitory action of bacteriocins is greatly influenced by intrinsic factors in food such as pH, lipid content, presence of proteolytic enzymes, and liquid versus solid systems, as well as by cell concentration (Ray and Daeschel, 1992). The presence of di- and tri-valent cations, such as Mg<sup>2+</sup>, Ca<sup>2+</sup> or Gd<sup>3+</sup>, significantly reduces the inhibitory activity of nisin Z against *Listeria monocytogenes* (Abee *et al.*, 1994).

#### **1. pH**

The pH of the environment not only affects the solubility of nisin but also its susceptibility to heat and antimicrobial activity. At pH 8, the solubility of nisin is only 0.25 mg/ml whereas 57 mg/ml are soluble at pH 2 (Liu and Hansen, 1990). Nisin undergoes a chemical modification and loss of activity at higher pH. However, this response is reversible

if the pH is decreased (Hurst, 1981; Liu and Hansen, 1990). Hurst (1981) demonstrated that nisin could be autoclaved without loss of activity at pH 2. Other investigators have shown that as pH increases there is a decrease in nisin stability and solubility (Hurst, 1981; Liu and Hansen, 1990). Optimum stability of nisin concentrates occurs at pH 3.0. Less than 5% nisin activity is lost after heating at 115°C for 20 minutes at pH 3.0. If the pH is reduced to 2.0, 28.5% of nisin activity is lost, while 21.4% is lost at pH 4.0 (Davies *et al.*, 1998).

In a chocolate milk model system (CMMS), *Bacillus stearothermophilus* spores were more heat sensitive when exposed to nisin (0, 2000 and 4000 IU/ml) in more acidic environment. When the pH was 5.7, the  $D_{130C}$  values were 15.5, 11.3, and 10.4 seconds versus 8.6, 5.6 and 5.0 seconds, respectively, when the pH of the CMMS was 5.0 (Beard *et al.*, 1999). Nisin A and Z demonstrated an increase in inhibitory activity against bacteria at acidic pH values and were capable of permeabilizing cell membranes at very low or absent membrane potentials (Abee *et al.*, 1994; Gao *et al.*, 1991, Garcia-Garcera *et al.*, 1993).

## 2. Fat content

Studies have shown that increasing concentrations of fat in foods result in decreased nisin activity against bacteria (Daeschel, 1990; Dean and Zotolla, 1996; Jung *et al.*, 1992). In the presence of 13% fat, nisin activity decreased by about 88%. Moreover, nisin exerted a greater inhibitory effect against *L. monocytogenes* in ice cream containing 3% fat verses ice cream containing 10% fat (Dean and Zotolla, 1996). In a study involving a chocolate milk model system containing either 10-12% or 0-1% fat, nisin was evaluated at 0, 2000 and 4,000 IU/ml. Under these conditions, the  $D_{130C}$  value decreased from 21.7, 17.2 and 17.8 seconds (10-12% fat) to 18.7, 14.0 and 13.9 seconds (0-1%fat), respectively (Beard *et al.*, 1999).

The reason for this loss of inhibitory activity of nisin with increasing fat content is not yet fully understood. The presence of fat in milk products may protect the spores because of the poor heat conductivity of lipids (Lang, 1935). Spores may also be protected from heat by increasing the concentration of free fatty acids in the lipids (Molin and Snygg, 1967). Another theory is that fat may bind to nisin thus preventing it from reacting with spores or vegetative cells (Delves-Broughton, 1990). Moreover, since nisin is a hydrophobic molecule, it is more attracted to lipid components in foods and therefore may not be evenly distributed in foods, thus leading to a reduction in antibacterial activity (Ray, 1992).

The results of several other studies demonstrated that nisin and nisin with EDTA are less effective against Gram negative bacteria when the organisms are attached to beef tissue surfaces than when cells are suspended in broth (Cutter and Siragusa, 1995a; Cutter and Siragusa, 1995b). The authors suggested five possible reasons for this occurrence. The first possibility is that nisin may be degraded by endogenous proteases present in the meat. Second, nisin may preferentially bind to adipose tissue resulting in an heterogeneous distribution of nisin across the tissues. The solubility of nisin may also decrease at the near neutral pH of meats. The presence of other ions on meat surfaces may interfere with the binding of EDTA to magnesium in the bacterial outer membrane lipopolysaccharide layer. Finally, nisin easily binds to meat proteins reducing their availability to bind with bacterial cells (Cutter and Siragusa, 1995a; DeVuyst, 1994).

### 3. Protein content

While some researchers believe nisin will bind to proteins thus reducing its activity against bacteria, a study by Stevens *et al.* (1992) who examined the effects of added protein on nisin activity proved otherwise. They compared the effects of adding 1mg/ml of bovine

serum albumin to each of the following four treatments: (i) cell buffer, (ii) 20 mM EDTA in cell buffer, (iii) 50 µg/ml nisin in cell buffer and (iv) 20 mM EDTA and 50 µg/ml nisin in cell buffer. Although the added protein was 20 times the concentration of nisin, no reduction in nisin/EDTA activity was detected against six *Salmonella* serotypes.

#### 4. Temperature

Temperature also effects nisin activity. The rate of Nisin Z-induced K<sup>+</sup> efflux from *L. monocytogenes* cultured at 30°C was greatly reduced at decreased temperatures. This finding is probably due to the ordering of hydrocarbon chains in cell membranes that occurs at lower temperature, thus decreasing the membrane fluidity (Abee *et al.*, 1995; Hurst, 1981; Liu and Hansen, 1990).

Many investigations have focused on using nisin to reduce the heat resistance of bacterial spores (Campbell and Sniff, 1959; Fowler and McCann, 1971; Heinemann *et al.*, 1964; O'Brien *et al.*, 1956). Indeed, nisin has been shown to reduce the thermal process requirements in some foods by increasing the thermal inactivation rate of bacterial spores and vegetative cells (Delves-Broughton, 1990). In a chocolate milk model system (CMMS) containing 10-12% fat cocoa powder and 0, 2,000 or 4,000 IU of nisin/ml, a 3 to 4 log reduction in *Bacillus stearothermophilus* population was observed in less than 80.0 seconds when heated at 130°C. The rate of spore inactivation increased (0.0549 to 0.0796 s<sup>-1</sup>) as the concentration of nisin increased. After five hours of exposure to nisin in the CMMS (pH 6.4), the D<sub>130C</sub> values were 21.7, 17.2, and 17.8 seconds for the 0, 2,000, and 4,000 IU/ml nisin treatments, respectively (Beard *et al.*, 1999). D<sub>130C</sub> values continued to decrease as exposure time increased. After 24 hours of exposure to nisin, D<sub>130C</sub> values were 18.7, 14.0, and 13.9 seconds, respectively.

Stevens *et al.* (1992) demonstrated that when Gram negative organisms were suspended for 60 minutes in 50 µg/ml nisin and 20 mM EDTA (pH 6.5), larger population reductions were observed as exposure temperature increased. For example, when cell suspensions were incubated at 4°C, significantly lower population reductions were observed than at higher temperatures of 20°, 30°, 37° and 42°C.

When *E. coli* O157:H7 ATCC 43894 cells were initially heated at 50°C for 15 min and then plated on tryptic soy agar containing 100 IU/ml nisin, the population was reduced from 6.3 to 3.9 log cfu/ml after 48 hours of incubation (Lee *et al.*, 2002). The more severe (higher temperatures, longer exposure) the initial heat treatment, the greater the effect of nisin. For example, when *E. coli* O157:H7 cells were first heated at 55°C for 10 and 15 min and then incubated at 37°C for 6 hours, a nisin concentration of 100 IU/ml nisin completely eliminated the cells (Lee *et al.*, 2002).

## 5. Usage

Bacteriocins can act as natural preservatives in many fermented dairy and meat products due to their inhibitory properties (Bender and Brodsky, 1990). Since food ingredients generally protect nisin from heat during pasteurization and sterilization processes (Heinemann *et al.*, 1965), the protein can be added to food products that undergo thermal processing. For example, bacteriocins can be added as an initial hurdle step in the production of thermally processed shelf-stable chocolate milk. This approach may mean that less severe heat treatments may be required if the heating step is preceded by the addition of bacteriocins (Beard *et al.*, 1999; Fowler and McCann, 1971; Heinemann *et al.*, 1964). By reducing the heating time, some food products would have improved quality and nutritional value, not to

mention lower processing costs and longer run times between cleaning (Gregory *et al.*, 1964).

Some studies have focused on using nisin in dairy products to reduce the population or heat resistance of bacterial spores (Campbell and Sniff, 1959; Gould, 1964; Heinemann *et al.*, 1964; O'Brien *et al.*, 1956). Indeed, nisin has been shown to reduce the thermal process requirements for some foods by enhancing the thermal inactivation of bacterial spores and vegetative cells (Delves-Broughton, 1990). Other applications of nisin as a food-preserved include shelf-life extension of dairy products as well as spoilage prevention in canned foods (Hurst, 1978). Nisin has also been evaluated as a food preservative in cottage cheese, eggs, brandy and canned foods (Benkerroum and Sandine, 1988; Delves-Broughton *et al.*, 1992; Gibbs and Hurst, 1991; Henning *et al.*, 1986). By adding nisin to cured meats the concentration of nitrites can be reduced (Rayman *et al.*, 1981). Other successful uses of nisin have also been in high pH/heat-treated food products including canned vegetables and pasteurized liquid eggs (Delves-Broughton, 1990; Delves-Broughton *et al.*, 1992).

## **VII. Dissertation Research Objectives**

While food processors in the United States and many other industrialized countries have implemented HACCP safety plans into their processing plants, millions of pounds of ready-to-eat meat products are still annually withheld or recalled from retail stores when microbial test results indicate the presence of pathogenic microorganisms. Even though many safety measures are applied, numerous food borne illnesses occur annually due to *Campylobacter jejuni* and *Escherichia coli* O157:H7. In order to prevent the loss of food products and human illnesses due to contaminated food products, the food industry is interested in identifying additional effective critical control steps to prevent or reduce food

contamination. Since many ready-to-eat products are pre-cooked, all pathogenic microorganisms should be destroyed. However, recontamination can occur when the food product is sliced or handled prior to packaging. By introducing an additional heat kill step following packaging, no product recontamination should occur until the package is opened, thus ensuring the consumer will receive a safe product. The first objective of this research was to determine the decimal reduction times (D-) and  $Z_D$ -values of *C. jejuni* and *E. coli* O157:H7 when inoculated on the surface of a low-fat turkey bologna packaged in a multilaminate film. While heat inactivation data of this type is already available in the literature for *C. jejuni* and *E. coli* O157:H7, the thermal lethality kinetics of these two organisms are dependent on the food product and heat inactivation methods employed. The second objective of this study was to determine if wheat gluten films containing nisin, citric acid, EDTA and Tween 80 effectively inhibit *C. jejuni* and *E. coli* O157:H7 when each organism was suspended in 0.1% peptone water. Data collected from the two previous studies were used in a third study to determine whether a combined mild surface heat pasteurization step and inclusion of inhibitory films composed of wheat gluten and glycerol with nisin, citric acid, and Tween 80, would significantly inhibit these two pathogens on vacuum-packaged, low-fat turkey bologna. By incorporating these agents into the packaging material it was hypothesized that a less severe heat treatment could be applied yet still achieve the desired level of kill for these pathogens. Moreover, since the antimicrobial agents were expected to migrate over time from the packaging films onto the surface of the product, we hypothesized that they would be present throughout the product's storage life and therefore would provide a safer and longer shelf life product.

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**MANUSCRIPT 1**

**Decimal Reduction and  $Z_D$ -Values of *Escherichia coli* O157:H7 and *Campylobacter jejuni* in Packaged Low-Fat Ready-To-Eat Turkey Bologna Subjected to a Surface Pasteurization Treatment<sup>†</sup>**

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## ABSTRACT

Cross-contamination of ready-to-eat (RTE) luncheon meat with foodborne pathogens can occur post-processing prior to packaging. Because these types of products often receive no additional heating prior to consumption, they constitute some degree of foodborne disease risk for consumers. To reduce this risk it may be feasible to apply a water immersion, surface pasteurization process following packaging. The objective of this study was to derive the decimal reduction times ( $D$ -values) and  $z_D$ -values of *Escherichia coli* O157:H7 (a mixture of strains HC 1840, HC 19486, HC 766 and ATCC 43894) and *Campylobacter jejuni* (a mixture of strains USDA-ARS WCR 72927, WCR 71940, and EPI 36) following inoculation on the surface of lean turkey bologna samples (66.08% moisture, 13.76% fat, 11.00% protein, 6.45% carbohydrate, 2.71% ash). Inoculated 3.6 cm<sup>2</sup> bologna samples (three physically separated, single layer samples per package) were vacuum packaged in 22.0 x 15.3 cm Cryovac P64x pouches (2.6 mil, a multilaminate film composed of nylon skin and barrier layers and a linear low-density polyethylene sealant layer) and immersed in a circulating water bath for predetermined lengths of time.  $D$ -values for *E. coli* O157:H7 were calculated from survivor plots at postpackage pasteurization temperatures of 55°, 60°, 65°, 70° and 75°C. Surviving organisms were plated on Sorbitol MacConkeys agar (37°C, 24-48h).  $D$ -values were 289.5, 45.8, 15.8, 11.9 and 9.1 seconds, respectively ( $z_D$ -value = 13.9°C). *C. jejuni* was recovered on Campy Cefex agar (42°C, 48h, microaerophilic environment).  $D$ -values for *C. jejuni* at 53°, 55°, 60°, and 62°C were 272.0, 192.1, 38.4, and 25.2 seconds, respectively ( $z_D$ -value = 8.3°C). These findings indicate that *C. jejuni* has a greater temperature dependence (*i.e.*, more heat sensitive) than *E. coli* O157:H7. The results of these postpackage pasteurization studies provide the time and temperature parameters

necessary to develop water immersion-based surface pasteurization processes for RTE deli meats such as bologna.

## INTRODUCTION

Over the past thirty years the per capita consumption of chicken has more than doubled in the U.S., reaching 54 lb (24.5 Kg) in 1999 (45). This increase is due in part to health-related concerns associated with red meat products and the development of new convenient further processed poultry products (45). The poultry industry began marketing more cut-up and further processed products in the 1950's and 60's, which included the use of mechanically separated meat from carcass parts such as frames, necks, and backs. Mechanically separated poultry meat is used in producing such emulsified products as bologna, salami, and frankfurters (19). These ready-to-eat (RTE) products are formulated by blending together skeletal muscles, mechanically deboned poultry or turkey meat, other protein sources, and other ingredients such as salts, sweeteners, and cure ingredients. After these ingredients are mixed together, the resulting batter is then vacuumed stuffed into a casing and thermally processed using a staged cook cycle, with final internal temperatures reaching 160 to 165°F (71.1 to 73.9°C). The cooking process is designed to eliminate all pathogenic bacteria that may be present in the raw ingredients. The cooked product is chilled to  $\leq 40^{\circ}\text{F}$  ( $\leq 4.4^{\circ}\text{C}$ ) and stored in coolers at 26 to 28°F (-3.3 to -2.2°C) before the casing is mechanically peeled from the frankfurters or sliced in the case of the bologna. Following these steps the products are then packaged and stored at 32°F or 0°F (0 or -17.8°C) before shipment to central distribution centers or retail outlets. It is during the slicing and peeling stage that these types of products are most susceptible to recontamination with pathogenic microorganisms (25). The equipment used in further processing plants such as slicers, dicers, and brining and packaging machines have been shown to harbor pathogenic bacteria, namely *Listeria monocytogenes* (5, 29, 44). Studies have shown that the bacterial loads in

commercially-produced RTE meat products can increase from  $\log_{10}$  0.5 to 2.0 cfu/g of product during the slicing process alone (23). Post-process handling is considered to be the primary cause of contamination of RTE meat and poultry products (13, 49).

In 2001, there were 13,705 laboratory-diagnosed cases of foodborne diseases caused by the seven bacteria and two parasites tracked under the foodborne disease surveillance FoodNet program. Of these nine foodborne disease agents, *Campylobacter* was ranked second (4,470 cases) to *Salmonella* (5,198 cases) as the most common cause of confirmed bacterial gastroenteritis in 2001 (10). Due to the high number of foodborne diseases, a major goal of public health agencies, food manufacturers and food service operations is the prevention of human illnesses caused by foodborne pathogens. This goal is being achieved by the use of HACCP-based food safety principles by many U.S. and foreign manufacturers (48). While *L. monocytogenes* is the most common pathogenic bacteria responsible for meat and poultry product recalls, other pathogens have been isolated from RTE foods (46).

Despite the fact that *E. coli* O157 accounted for only 4% of the confirmed cases of bacterial gastroenteritis in the US in 2001 (10), it is nevertheless of great concern because annually it causes an estimated 20,000 cases of foodborne illness resulting in approximately 250 deaths. In addition, it is the most common cause of hemolytic-uremic syndrome (HUS) in the U.S. (2). In 2000, it was estimated that the 62,458 reported foodborne illness cases involving *E. coli* O157:H7 had associated costs of \$659.1 million (approximately \$10,500/case) (46). Although ground beef is most often incriminated with outbreaks of *E. coli* O157:H7, other meat products have been linked to outbreaks as well. These include roast beef (35), salami (11), dry-cured salami (11), fermented salami (11, 43), venison jerky (24), and unrefrigerated sandwiches (9). To date, there have been no reported cases of

foodborne *E. coli* O157:H7 associated with poultry products. However, Doyle and Schoeni (17) isolated *E. coli* O157:H7 from 1.5% (4/263) of the poultry samples they sampled from retail stores in Wisconsin and Alberta, Canada. A second research group employing a shiga toxin gene probe reported 12% of the chicken and 7% of the turkey samples tested were positive for the shiga toxin (37). These two studies demonstrate the potential risk of many meat products including poultry products in contributing to foodborne illnesses involving *E. coli* O157:H7.

The infectious dose of this organism is believed to be relatively low. An investigation of an outbreak associated with dry-cured salami estimated the infectious dose of *E. coli* O157:H7 to be less than 50 organisms, with some of the cases having an estimated infectious dose of less than five organisms (43). Other investigators estimated the infectious dose of *E. coli* O157:H7 to be in the range of 2 to 45 bacteria in an outbreak involving fermented sausage (37). Hamburger patties incriminated in an *E. coli* O157:H7 outbreak in 1993 contained fewer than 700 organisms per patty prior to cooking; therefore, the actual microbial load should have been lower at the time of consumption (21). It is believed that there is a three- to four-day incubation period after ingestion of the organism. During this time the bacteria colonize the large intestine and subsequently multiply. While most sufferers have a non-bloody diarrhea and abdominal cramps, patients that seek medical attention typically have a bloody diarrhea. The bloody diarrhea is generally observed during the second or third day of the illness (33). The illness commonly lasts for one week, yet 6% of the patients develop the hemolytic-uremic syndrome (HUS) (20).

*Campylobacter* species are commensals in most animals, normally colonizing the gastrointestinal tracts of many different animals (12, 42). While this organism does not grow

under refrigeration conditions, it survives well in foods held at refrigeration temperatures (16). The infectious dose of *Campylobacter* spp. can be as low as 500 to 800 organisms. Symptoms of campylobacteriosis are generally detected two to five days after consumption of contaminated product (34). The illness may persist up to 10 days (7, 8). The disease generally starts with a fever, often over 40°C (104°F), with malaise, abdominal pains and headaches, followed by diarrhea. The patient's stool will become liquid to watery and often contain polymorphonuclear leukocytes (7). Acute diarrhea may last from two to three days with abdominal discomfort lasting for several additional days (8). In mild cases the organism can be isolated only up to a few days after the patient ceases to show symptoms, yet in more severe cases the organism has been isolated from feces of patients two to seven weeks after symptoms have subsided (3).

Post-processing contamination of RTE meat and poultry products remains a serious public health threat since these products are generally not reheated prior to consumption. In 2000, class I product recalls of further processed RTE meat products suspected of being tainted with pathogenic bacteria amounted to over 35 million pounds of hot dogs and 14.5 million pounds of turkey and chicken delicatessen meats (47). To prevent future recalls of poultry and meat products, studies are underway to establish if a post-package pasteurization process might be a feasible means of eliminating product contamination occurring before or during packaging. Some of these studies have tested microwave heating (38), steam (15) and hot water (13, 14, 36) as a means to achieve pathogen-free products.

The objective of this study was to kinetically characterize the heat resistance (*i.e.*,  $D$ - and  $z_D$ -values, activation energies, rate of inactivation) of *E. coli* O157:H7 and *C. jejuni* on the surface of packaged low fat turkey bologna samples pasteurized at temperatures of 55°,

60°, 65°, 70°C, and 75°C or 53°, 55°, 60°, and 62°C, respectively. This kinetic information will be beneficial to poultry and red meat processors interested in exploring post-package pasteurization of RTE deli meats. This study is part of a comprehensive investigation with colleagues from Clemson University who are conducting similar studies with *Listeria monocytogenes* and *Salmonella* Typhimurium.

## MATERIALS AND METHODS

**Bacterial cultures.** The *E. coli* O157:H7 culture strains used in this study (and their sources) included HC 1840 (human isolate), HC 19486 (human isolate), and HC 766 (human isolate) obtained from the Bureau of Microbial Hazards, Health and Welfare Canada, Ottawa, Ontario, Canada; and ATCC 43894 (human isolate), obtained from the American Type Culture Collection (Rockville, MD). *Campylobacter jejuni* strains WCR 72927, WCR 71940, and EPI 36 were obtained from Dr. Eric Line, USDA-ARS (Athens, GA).

**Culture maintenance and inoculum preparation.** Stock cultures of *E. coli* O157:H7 strains were maintained in sterile double strength brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) and glycerol (50:50 vol) and stored at -20°C. A 0.1% (vol/vol) inoculum of each stock culture was used to prepare a fresh BHI culture (37°C for 24 h with agitation). For each *E. coli* O157:H7 strain, 10 µl samples of the broth culture were transferred to 10 ml of fresh BHI broth and incubated at 37°C for 24 h with agitation. The 10 ml cultures were pooled together (four strains) and centrifuged at 12,000 X *g* for 12 minutes at 5°C. The pellet was washed twice in 0.1% peptone water, centrifuged again as previously described, and resuspended in an appropriate volume of 0.1% peptone water to obtain a targeted population of approximately log<sub>10</sub> 9.0 cfu/ml.

*Campylobacter jejuni* strains were maintained on Campy Cefex agar (Gibson Laboratory, Lexington, KY) at 4°C and then transferred to Brucella broth (Difco Laboratories, Detroit, MI) 4-days prior to initiating an experiment (42°C for 48 h in a microaerophilic gas environment of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>). Thirty µl of each test culture (0.1% inoculum, vol/vol) were transferred to 30 ml of fresh Brucella broth and incubated as previously described. The 30 ml cultures were pooled together, centrifuged, washed twice in 0.1% peptone water, and resuspended in 0.1% peptone water according to the procedures described for *E. coli* O157:H7. The targeted population was approximately log<sub>10</sub> 9.0 cfu/ml.

**Bologna.** A low fat turkey bologna averaging 14.2% fat, 10.7% protein, 6.45% carbohydrate, and 2.71% ash was obtained from a commercial source, cut into 4 cm<sup>2</sup> samples, packaged in Cryovac P64x pouches (Cryovac Inc., Duncan, SC), and frozen before being irradiated with a 10kGy dose for 79 minutes using a Cobalt 60 source (at Auburn University, AL). Samples were irradiated to significantly reduce the background microflora but not to necessarily produce sterility. The background microflora populations following irradiation ranged from 1.5 to 13.5 cfu/gm of bologna. Irradiated bologna samples were stored at -20°C and thawed for 24 h at 4°C prior to initiating an experiment.

**Determination of heat resistance.** Resuspended cells of each pooled organism in 0.1% peptone water (25 µl) were aseptically spread across the top surface of each irradiated bologna sample using a sterile L-shaped glass rod and allowed to air dry (around 15 minutes, target populations were log 7.4 and 8.2 cfu/sample for *E. coli* O157:H7 and *C. jejuni*, respectively). Following inoculation, three samples of bologna were aseptically transferred to a 22 cm x 15.3cm, 2.6 mil multilaminate pouch composed of nylon skin and barrier layers

and a linear low-density polyethylene sealant layer (P64X pouch, oxygen transmission rate of 60cc/m<sup>2</sup>/day at 23°C and 0% relative humidity, Cryovac Inc., Duncan, SC) and then each vacuumed sealed (one single layer of product) in three separate chambers per pouch. The sealed pouches were immersed in a preheated circulating water bath equipped with a calibrated temperature control module accurate to  $\pm 0.05^{\circ}\text{C}$  (Model DC1, Haake, Inc., Karlsruhe, Germany). Using a type K thermocouple probe linked to a digital thermometer (model 871, Omega Engineering Inc., Stamford, CT), the heating (come-up) period required for the bologna sample surface to reach that of the water bath ranged from 27 to 103 s. At pre-selected time intervals, a pouch containing three separated bologna samples was removed from the water bath and rapidly cooled for 10 minutes by immersion in an ice-water slurry. The sample surface temperature was monitored over the course of heating and cooling (1 second intervals) using a type K thermocouple attached to a Tempest Datalogger (Tangent Systems, Inc., Charlotte, NC). Typical sample temperature profiles at each water bath temperature are presented in Figures 1 and 2 for *E. coli* O157:H7 and *C. jejuni*, respectively. The three bologna samples were aseptically removed from each pouch and placed into separate stomacher filter bags (Spiral Tech, Norwood, Massachusetts) containing 5 or 10 ml of 0.1% peptone water and blended for one minute using a IUL Masticator (IUL Instruments, Cincinnati, OH). This initial dilution was then serially diluted in 0.1% peptone water as needed, and surface-plated onto Sorbital MacConkey Agar for *E. coli* O157:H7 (37°C for 24 h, Gibson Laboratory, Lexington, KY) or Campy Cefex agar for *C. jejuni* (42°C for 48 h, Gibson Laboratory, Lexington, KY). Campy Cefex agar plates were placed in freezer bags (Ziploc, S.C. Johnson and Son Inc., Racine, WI) and then incubated for 48h in a

microaerophilic environment as previously described. Following incubation, colonies were enumerated for each temperature and trial.

**D-value and  $z_D$ -value determinations.** Triplicate thermal inactivation trials were conducted for each pooled test strain at 55°, 60°, 65°, 70°, and 75°C for *E. coli* O157:H7 and 53°, 55°, 60°, and 62°C for *C. jejuni*. Survivor curves (log viable *E. coli* O157:H7 or *C. jejuni* per ml versus time) were plotted for each temperature and trial, and best fit linear regression lines were determined using a personal computer and a graphics/statistics program (Microsoft 2000 Excel, Redmond, Wash.). *D*-values (seconds) were calculated as the negative reciprocal of the survivor curve slope obtained by regression analysis. Decimal reduction time curves (mean log *D*-value versus temperature) were also constructed for each bacteria type, and  $z_D$ -values were calculated, where  $z_D = \text{slope}^{-1}$  (i.e., the temperature change [°C] necessary to effect a 10-fold change in the *D*-value). The *k* value or rate of inactivation at a tested temperature was calculated as the negative slope of the  $\ln(N/N_0)$  (the fraction of survivors where *N* = the population at time *t* and *N*<sub>0</sub> = the population at *t* = 0) in contrast to heating time plot at each tested temperature and could be estimated as  $2.303/D$  (32). The activation energy of thermal inactivation (*E*<sub>a</sub>) was calculated from the negative slope of the  $\ln(k)$  in contrast to inverse temperature (1/*T*) plot. The Arrhenius constant (*a*) was calculated from the intercept of the  $\ln(k)$  in contrast to 1/*T* regression. Slopes and *r*<sup>2</sup> values were determined using least square linear regression.

**Statistical analysis.** At each target temperature, mean decimal reduction times obtained via the bologna heat inactivation studies were compared within bacterial types using the general liner model procedure of SAS where the model included temperature, replication,

and temperature by replication as the main effects (Version 8.0. Statical Analysis System, Cary, NC).

## RESULTS AND DISCUSSION

**Raw product composition.** The total water content of the turkey bologna averaged 66.1% (w/w, wet basis), using an oven drying method at 101 to 102°C for 16 to 18 h. The total protein content averaged 11.0% (w/w, wet basis) using the Kjeldahl method. The total lipids content averaged 13.8% (w/w, wet basis, Soxhlet method) whereas the total ash content averaged 2.7% (w/w, wet basis, 525°C for 24h) (4).

**Thermal profiles.** The thermal history (temperature recorded in 1 second intervals) of the bologna samples was monitored throughout the surface pasteurization process using type K thermocouples placed at the interface between the bologna surface and the interior of the vacuum-sealed pouch (Figs. 1 and 2). The heating lag time required for the sample surface to reach the water bath temperatures ranged from 27 to 103 seconds depending on the water bath temperature. The entire temperature history of the samples including come-up and cooling time was taken into consideration in the calculation of the kinetic parameters. The samples dropped below 20°C in less than 15 seconds during the cool down period.

**Survival of *E. coli* O157:H7 and *C. jejuni* on pasteurized bologna.** Analysis of uninoculated control samples revealed no *E. coli* O157:H7 or *C. jejuni* initially present on the irradiated bologna samples. The initial background bacterial populations ranged from 1.5 to 13.5 cfu/g of sample. The target inoculum populations ranged from log<sub>10</sub> 6.2 to 7.8 and 6.5 to 6.7 cfu/sample for *E. coli* O157:H7 and *C. jejuni*, respectively.

Typical survivor curves for *E. coli* O157:H7 (Fig. 3) and *C. jejuni* (Fig. 4) at the temperatures tested were constructed by plotting cfu/ml of masticated sample versus heating time. As expected, as heating temperature increased from 55 to 75°C for *E. coli* O157:H7 and 53 to 62°C for *C. jejuni*, survival of each organism decreased. Semilogarithmic survivor curves showed a linear logarithmic decline in population over heating time ( $r$  values ranged from 0.925 to 0.999) across both organisms, processing temperatures and triplicate replicates). Moreover, little or no evidence of shouldering was detected in the survival plots. Thus, mean  $D$ -values could be reliably calculated from this set of data. Table 1 documents the mean  $D$ -values and other thermal inactivation kinetic values for *E. coli* O157:H7 and *C. jejuni*. For *E. coli* O157:H7,  $D$ -values ranged from 9.1 seconds at 75°C to 289.5 seconds (4.82 min) at 55°C. Likewise,  $D$ -values ranged from 25.2 seconds at 62°C to 272.0 seconds (4.53 min) at 53°C for *C. jejuni*. Significant differences ( $P < 0.05$ ) in the  $D$ -values were observed for both test organisms due to process temperature. In order to provide a margin of safety in this study, a mixed pool of 3 to 4 strains of each organism was used. Construction of decimal reduction time curves for *E. coli* O157:H7 (Figure 5,  $r^2 = -0.855$ ) and *C. jejuni* (Figure 6,  $r^2 = -0.992$ ) yielded  $z_D$ -values of 13.9° and 8.3°C, respectively (Table 1).

The thermal resistance data for *E. coli* O157:H7 on bologna were similar to the results reported by Doyle and Schoeni (16) who reported  $D$ -values of 270, 45, and 9.6 seconds at 57.2°, 60°, and 64.5°C, respectively, and a  $z_D$ -value of 5.3°C for *E. coli* O157:H7 heated in ground beef. In comparison, we calculated  $D$ -values of 289.5, 45.8, and 15.8 seconds at process temperatures of 55°, 60°, and 65°C, respectively, and a  $z_D$ -value of 7.9°C for these three process temperatures. When all five  $D$ -values were used in calculating the  $z_D$ -value, the value nearly doubled to 13.9°C. This  $z_D$ -value is from 2.3 to 3.4 times higher than

the 4° to 6°C range previously reported for most non-spore-forming bacteria (22). This significant increase is attributed to the similar  $D$  values detected at 65°, 70°, and 75°C. In other studies, Kotrola and Conner (27) reported  $D$ - and  $z_D$ -values for *E. coli* O157:H7 in a low and high fat ground turkey product ranging from 38.5 min at 52°C to 54 seconds at 60°C and a  $z_D$ -value averaging 4.43°C. The variations in  $D$ - and  $z_D$ -values between studies may also be related to the differences in meat products evaluated (*i.e.*, a ground beef product with *E. coli* dispersed throughout the sample compared to a surface inoculation of a comminuted low fat turkey bologna product). In contrast to these previous studies involving meat, Steenstrup and Floros (40) derived the  $D$ - and  $z_D$ -values for *E. coli* O157:H7 in heated apple cider as a function of pH and preservative concentration. The  $D$ - and  $z_D$ -values ranged from 6.6 seconds (53°C) to 35.64 min (47°C) and 6.0° to 26.5°C, respectively.

Because of its fastidiousness under laboratory conditions, there is less reported information on the  $D$ - and  $z_D$ -values of *C. jejuni*. Blankenship and Craven (6) reported  $D$ -values at 49°, 51°, 53°, 55° and 57°C for *C. jejuni* strain H-840 suspended in 1% peptone water of 15.2, 4.9, 1.71, 0.64, and 0.25 min, respectively. The same researchers reported  $D$ -values for *C. jejuni* at the same temperatures to be 20.5, 8.77, 4.85, 2.12 and 0.79 min, respectively, when inoculated in ground chicken. The  $D$ -values they obtained at 53 ° (291 sec) and 55°C (127.2 sec) in ground chicken were similar to the  $D$ -values (272 sec and 192.1 sec, respectively) achieved in the present study at the same process temperatures. A  $z_D$ -value of 4.49°C was calculated from their  $D$ -value data. In the present study, a  $z_D$ -value of 8.3°C was calculated which is similar to the  $z_D$ -values reported for other foodborne pathogens such as *Listeria monocytogenes* (7.2 °C) in liquid whole eggs (18), *Salmonella* (6.53 °C) in ground chicken (32) and *Aeromonas hydrophila* (5.02-5.59°C) in liquid whole eggs (39). Direct

comparisons between studies are often not possible due to differences in the bacterial strains tested, the use of different food materials and packaging materials, and the methodologies used in the heat inactivation trials.

Kinetic inactivation rate constants ( $k$ , approximately  $2.303/D$ ) of  $0.48$  to  $15.15 \text{ min}^{-1}$  for *E. coli* O157:H7 and  $0.51$  to  $5.48 \text{ min}^{-1}$  for *C. jejuni* were obtained at the processing temperature ranges of  $55^\circ$  to  $75^\circ\text{C}$  and  $53^\circ$  to  $62^\circ\text{C}$ , respectively (Table 1). Compared to the lowest water bath temperatures tested for each organism ( $55^\circ$  and  $53^\circ\text{C}$ , respectively), *Escherichia coli* O157:H7 and *C. jejuni* cells were inactivated at a significantly higher rate (i.e., 32- and 11-fold, respectively) when heated at the highest water bath temperatures (i.e.,  $75^\circ$  and  $62^\circ\text{C}$ , respectively). The activation energy ( $E_a$ ) and the Arrhenius constant ( $a$ ) were  $105.3 \text{ kJ/mol}$  and  $6.70 \times 10^{16} \text{ min}^{-1}$ , respectively, for *E. coli* O157:H7 (Table 1, Figure 7) and  $254.2 \text{ kJ/mol}$  and  $2.37 \times 10^{40} \text{ min}^{-1}$ , respectively, for *C. jejuni* (Table 1, Figure 8).

Figures 9 and 10 represent the thermal death time curves generated to yield a 12- and 7-*D* surface heat pasteurization process for *E. coli* O157:H7 and *C. jejuni*, respectively. Due to *E. coli* O157:H7's relative pathogenicity, its high foodborne illness costs, and the seriousness of its foodborne illness complications, a 12-*D* process was calculated for this organism. The range of pasteurization times required to achieve a 12-*D* reduction in *E. coli* O157:H7 population on the surface of a low-fat turkey bologna was 1.8 min at  $75^\circ\text{C}$  to 58 min at  $55^\circ\text{C}$ . Because of the widespread incidence of *C. jejuni* on raw food products, especially raw poultry products, a 7-*D* thermal death time curve was plotted. Under these constraints, surface heat pasteurization times of 2.9 to 31.7 min would be required at  $62^\circ$  and  $53^\circ\text{C}$ , respectively.

Since the actual cooking process of RTE meat products is designed to eliminate bacterial pathogens, recontamination of such products is most likely to occur during the slicing/peeling process prior to packaging. Thus, if pathogens are present, they will generally be on the surface of the product. Because many of these foods are consumed without an additional heating step, there is some level of risk of food-borne illness associated with consuming these foods. To reduce these risks will require either a reduction in the cross-contamination potential prior to packaging or introduction of a control strategy such as post-packaging surface heat pasteurization. The application of flash pasteurization (15) and multiple cycles of vacuum and steam treatments (28) have been successfully tested on unpackaged frankfurters to reduce *Listeria monocytogenes* populations on the product surface. Cygnarowicz-Provost and colleagues achieved two 4-*D* processes by evacuating a chamber of a small pasteurizer for 15 sec prior to exposing the beef frankfurters to steam at 136°C for 32 sec or 115°C for 40 sec. The frankfurters were then quickly cooled by evacuating the steam for 10 sec producing a vacuum packaged product (15). Using an initial vacuum time of 0.1sec, a final vacuum time of 0.5sec, and a steam time of 0.1sec, a 1.73 log cfu/ml reduction in population was achieved with a steam temperature of 126°C versus a 2.39 log cfu/ml reduction when the steam temperature was set at 138°C (28). While these approaches may be effective, neither addresses the potential for product recontamination prior to packaging. In contrast, other investigators have been evaluating the efficacy of post-packaging surface pasteurization treatments on RTE meat products. For example, microwave heating (38) and immersion in hot water (13, 14) have been evaluated as a means to decontaminate the surface of RTE packaged foods. In an investigation by Cooksey *et al.* (13), pre-cooked chunks (50 g) of USDA beef strip loins inoculated with *Listeria*

*monocytogenes* were vacuum packaged in bags and submerged in 82°C water for 16 min prior to being cooled on ice and refrigerated at 4°C. This treatment reduced the *L. monocytogenes* populations on the surface of the beef chunks by 5-logs and by 6-logs in the surrounding broth. In a similar experimental design, these same investigators observed over an 85 day refrigerated storage period a reduction in the *Clostridium perfringens* vegetative population of 5-logs in the meat broth and 1.5-logs on the meat surface. This process also reduced the number of vegetative cells from spores to an undetectable level. The maximum number of vegetative cells detected in the broth of the pasteurized beef chunks was  $\log_{10}$  0.28 cfu/ml at 7 days, while the population of vegetative cells produced from spores in the broth of unpasteurized beef chunks ranged from  $\log_{10}$  2.58 to 3.21 cfu/ml throughout 65 days of storage (14).

The amount of fat, salt content, pH, polysaccharide concentration, and presence and concentration of curing agents and water in foods can also significantly influence the heat resistance of bacteria (41). Koidis and Doyle (26) and Ahmed (1) demonstrated that the same bacterial strain would have varying *D*-values depending on the type of food product. For example, the fat content of a food will significantly affect the survivability of bacteria against heat. Ahmed (1) reported *D*-values at 55°C for *E. coli* O157:H7 ranging from 0.38 min in a lean (3% fat) ground chicken product to 19.3 min in a 20% fat ground beef product. Moreover, Muriana *et al.* (30) demonstrated that the same strain of *L. monocytogenes* has variable *D*- and  $z_D$ -values when suspended in the purge fluid from several RTE deli meats formulated from different animal species. Using a standard glass capillary tube heating method, a four-strain cocktail of *L. monocytogenes* was suspended in the purge fluids of smoked turkey and yielded *D*-values of 416.7, 73.5, 25.4, and 9.3 seconds, respectively, for

heating temperatures of 62.8, 65.6, 68.3, and 71.1°C ( $z_D$ -value = 5.1°C). Higher temperature treatments (90.6 - 96.1°C) ranging in process times from 2 to 4 min were required to achieve greater than a 2-log reduction in the surface population on inoculated RTE deli-style meat products (5 to 12 lbs). While the chemical composition of these different purge fluids was not reported (i.e. fat, salt, curing agents), their findings demonstrate how different foods can affect the heat resistance of bacteria. They further noted that although the purge fluid composition may resemble the actual meat product composition, the  $D$ -values varied depending on whether the cells were suspended in the meat purge versus inoculated on the surface of the meat product. For example, after 20 seconds of immersion in a 71.1°C steam-injected water bath, they detected a 5-log reduction in *L. monocytogenes* population in a roast beef purge compared to a 4-log reduction on the surface of the roast beef product following 10 minutes of heating.

Not only does the food product type influence the degree of thermal inactivation of bacterial pathogens in post-packaging applications, but the type and thickness of packaging materials can also affect the level of inactivation. Murphy *et al.* (31) demonstrated that the heat-penetration rate of a vacuumed-packaged, uniformly shaped chicken breast meat product (0.5 mm thick) was significantly influenced by the polyethylene packaging film thickness, which in turn affected the rate of bacterial inactivation. They observed that heating time ( $p < 0.0001$ , 10-120s) and heating time by film thickness ( $p < 0.05$ , 0.0762 and 0.2032mm) significantly influenced the survival of *Salmonella* and *L. monocytogenes*. Thicker films had slower heating rates and thus lower lethality. After the meat samples had been heated at 68°C for 120 sec, more than a 2 log<sub>10</sub> difference in surviving populations

of *Salmonella* and *L. innocua* were detected between meat packaged in 0.0762mm versus 0.2032mm thick films.

In the present study, each slice of bologna was packaged and processed individually. Although this form of single-layer packaging is not used commercially for packaged bologna, the results of this study do provide important surface heat pasteurization kinetic values that will be of potential benefit to processors who market single layer RTE meat items that are primarily contaminated on the surface (i.e., fully cooked, further processed meat products containing intact muscles). While in-package pasteurization can reduce or eliminate pathogenic bacteria, it is important to conserve product quality. Therefore, it may be more feasible to combine surface heat pasteurization with other food safety hurdles to reduce either the heating time or temperature yet still achieve an equivalent lethality without compromising product quality.

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TABLE 1. D-values,  $Z_D$ -values and activation energies of inactivation for *Campylobacter jejuni* and *E. coli* O157:H7 on the surface of low-fat turkey bologna.

Pathogen	Process temperature (°C)	D-values (s) at indicated temperature (°C) <sup>a</sup>	$Z_D$ values (°C)	k, rate of inactivation (min <sup>-1</sup> )	$E_a^c$ (kJ/mol)	Arrhenius constant (a) (min <sup>-1</sup> )
<b><i>E. coli</i> O157:H7</b>	55	289.5 A <sup>b</sup>	13.9	0.477	105.3	6.70x10 <sup>16</sup>
	60	45.8 B		3.018		
	65	15.8 C		8.757		
	70	11.9 D		11.631		
	75	9.1 E		15.151		
<b><i>C. jejuni</i></b>	53	272.0 F	8.3	0.508	254.2	2.37x10 <sup>40</sup>
	55	192.1 F		0.719		
	60	38.4 G		3.598		
	62	25.2 H		5.483		

<sup>a</sup> Mean of three trials ( $n = 3$  samples per temperature and trial).

<sup>b</sup> Values within pathogen type and column followed by the same letter are not significantly different ( $P < 0.05$ ).

<sup>c</sup> Activation energy of thermal inactivation.

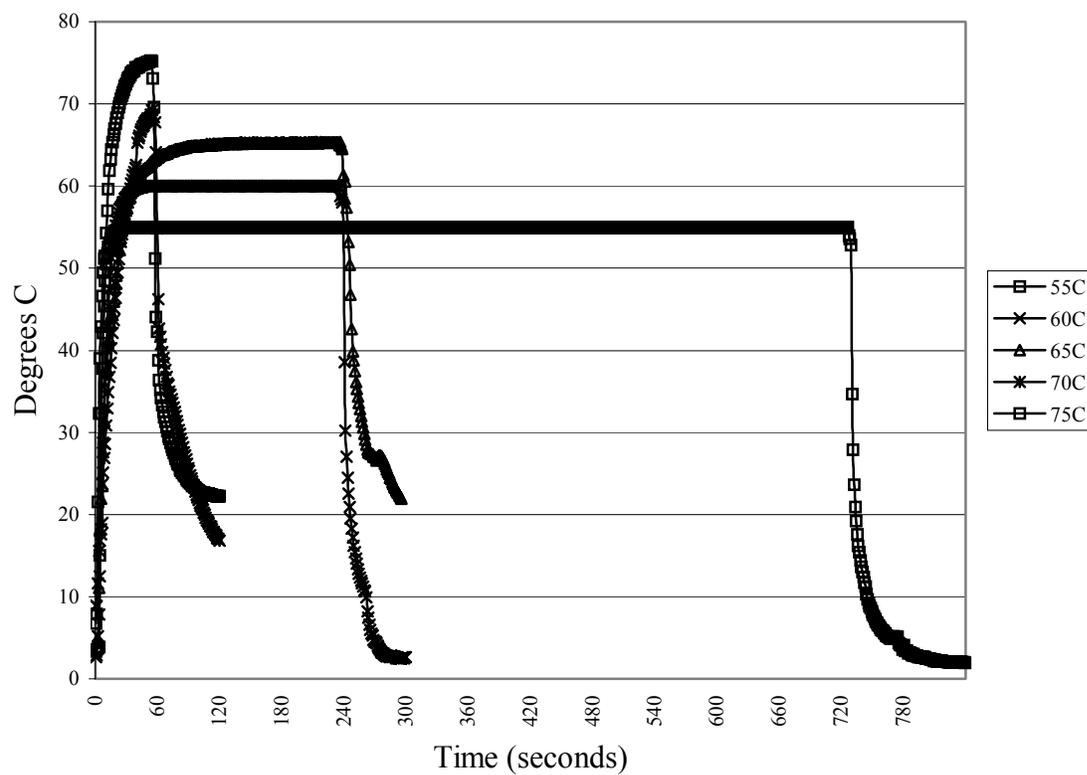
FIGURE 1. Typical bologna surface temperature profiles for *E. coli* O157:H7 trials.

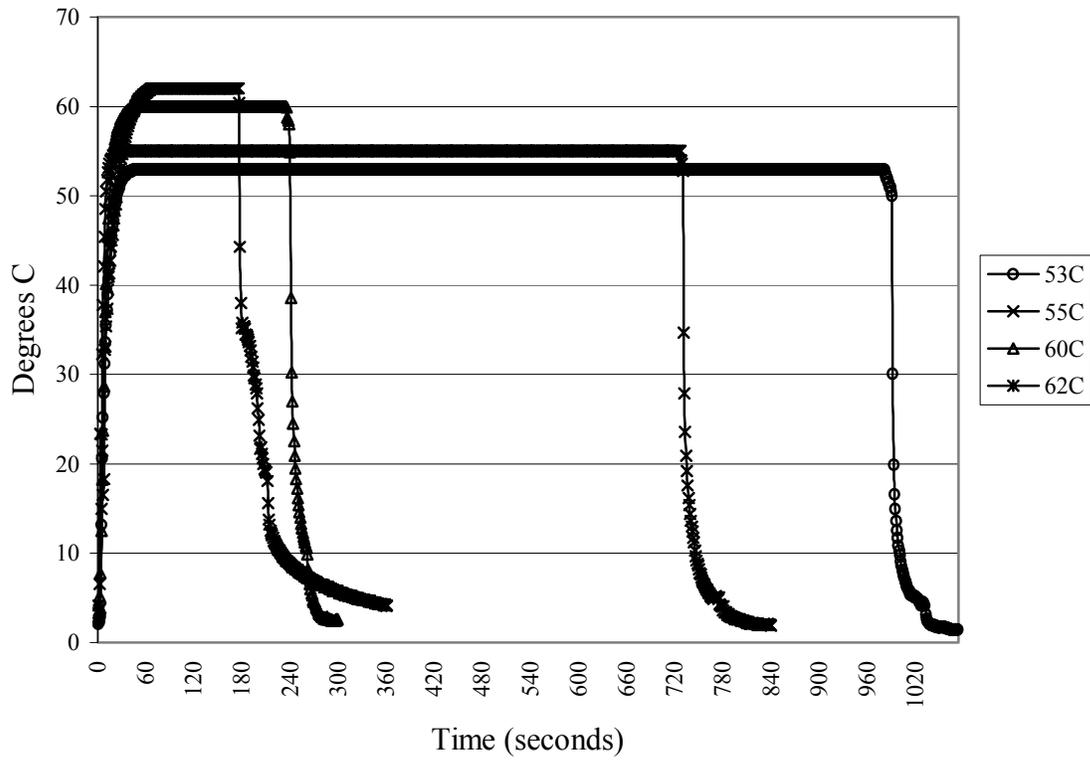
FIGURE 2. Typical bologna surface temperature profiles for *Campylobacter jejuni* trials.

FIGURE 3. Typical survivor curves of *E. coli* O157:H7 on bologna surfaces at different water bath temperatures (with trendline analysis equations).

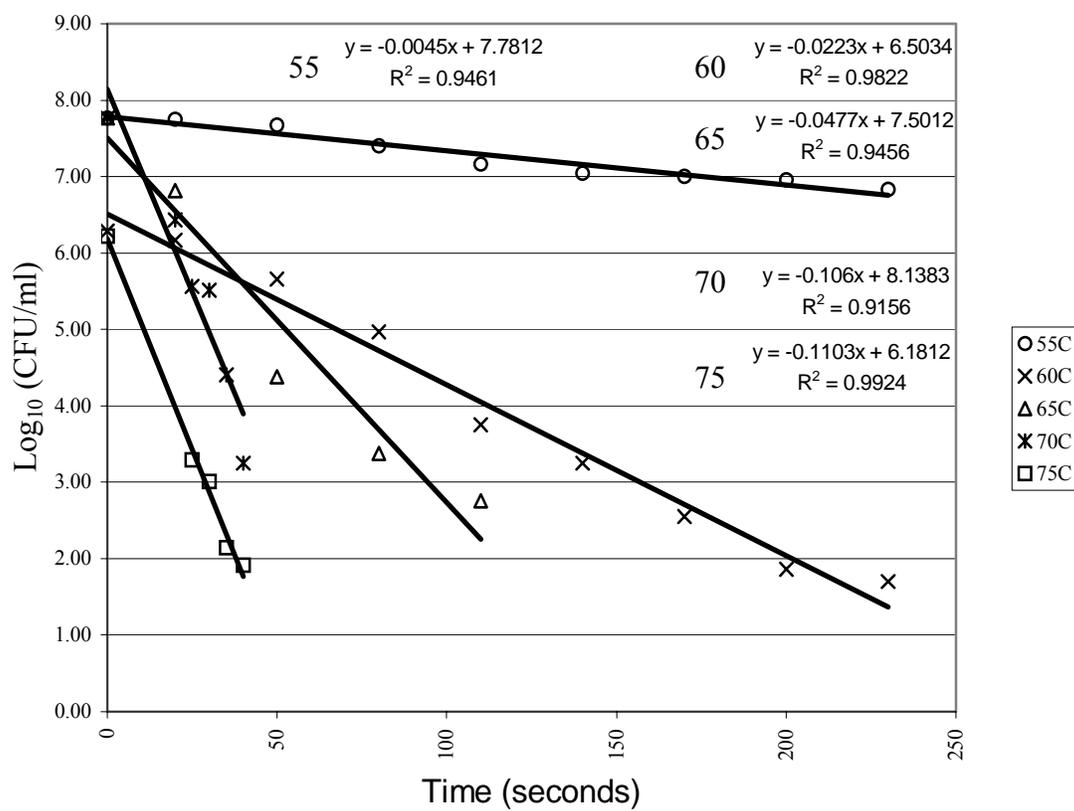


FIGURE 4. Typical survivor curves of *C. jejuni* on bologna surfaces at different water bath temperatures (with trendline analysis equations).

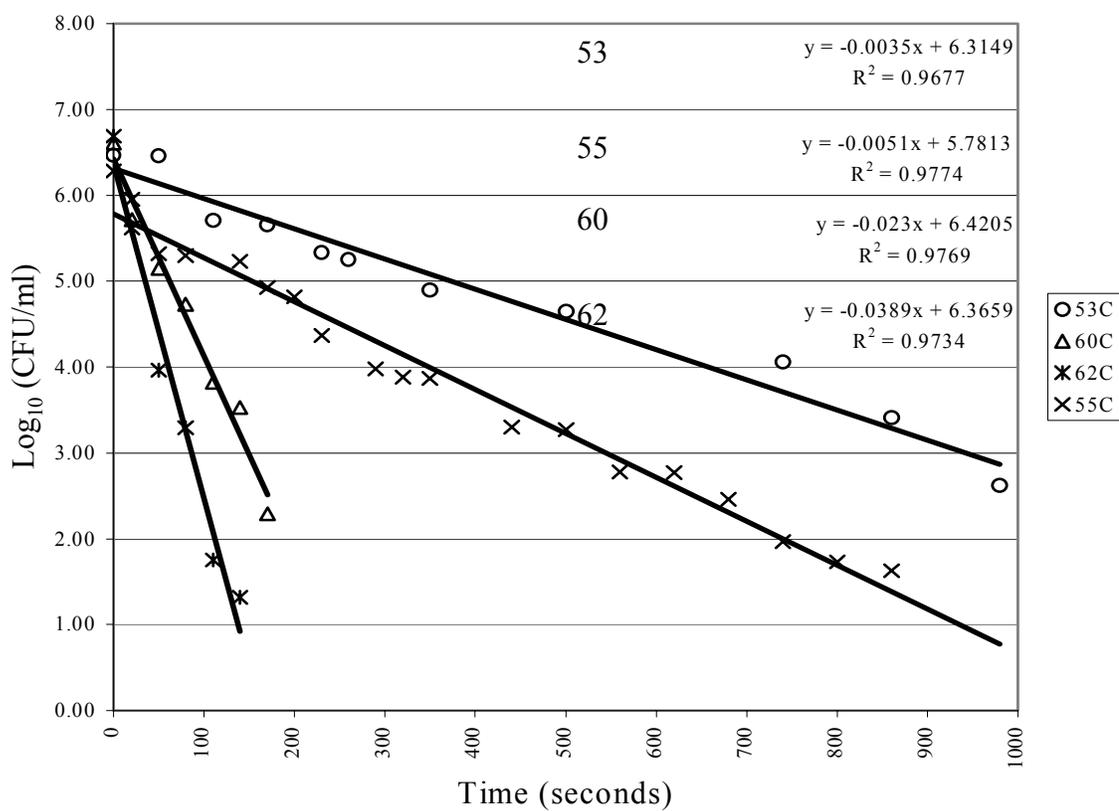


FIGURE 5. Logarithm of D-values for *E. coli* O157:H7 on bologna surfaces (with trendline analysis equation).

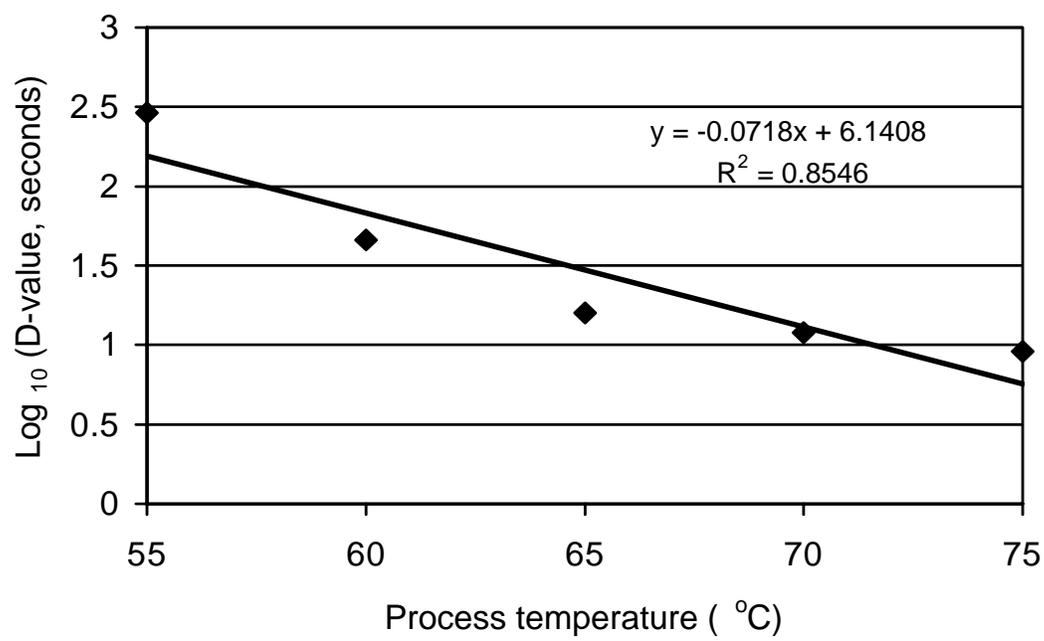


FIGURE 6. Logarithm of D-values for *C. jejuni* on bologna surfaces (with trendline analysis equation).

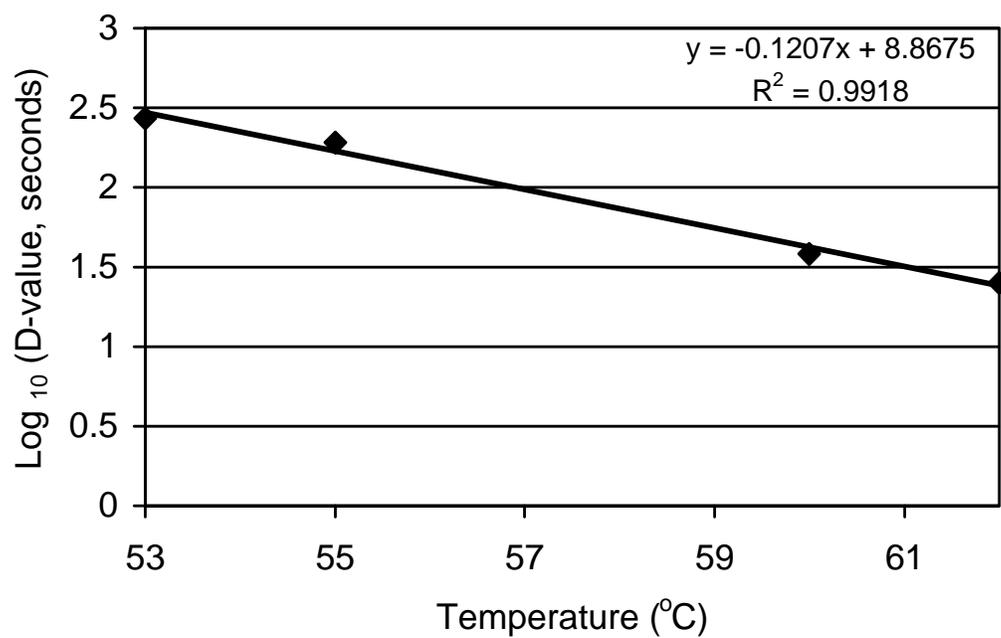


FIGURE 7. Natural logarithm of  $k$  ( $\text{min}^{-1}$ ) for *E. coli* O157:H7 on bologna surfaces at 55 to 75°C (with trendline analysis equation).

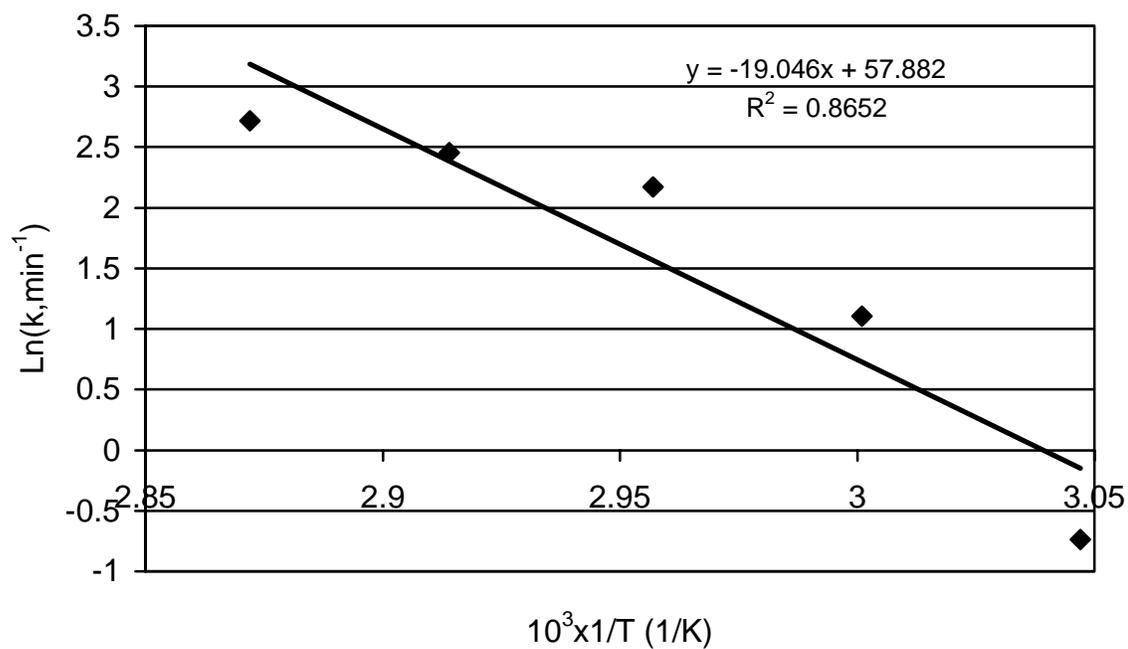


FIGURE 8. Natural logarithm of  $k$  ( $\text{min}^{-1}$ ) for *C. jejuni* on bologna surfaces at 53 to 62°C (with trendline analysis equation).

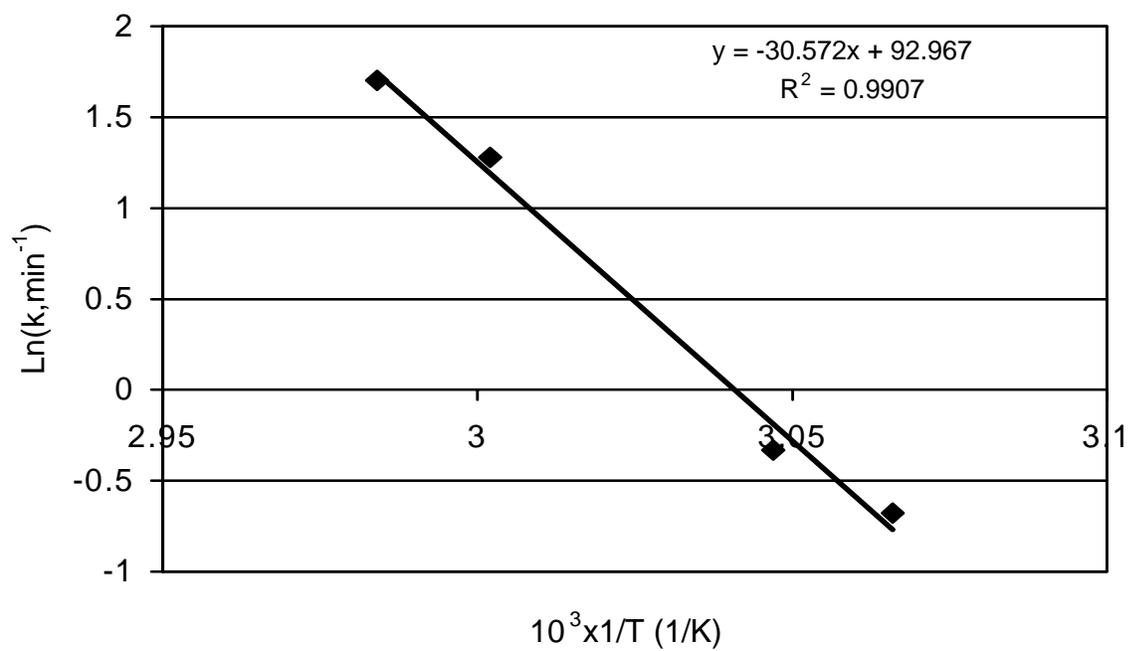


FIGURE 9. Thermal death time curve (12-D process) for *E. coli* O157:H7 on bologna surfaces (with trendline analysis equation).

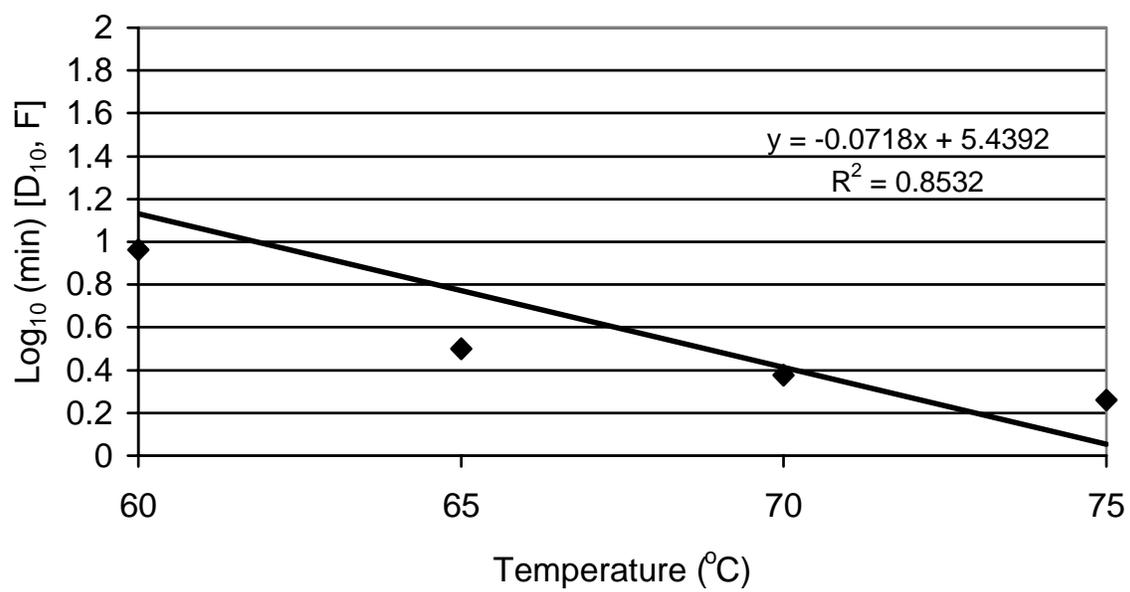
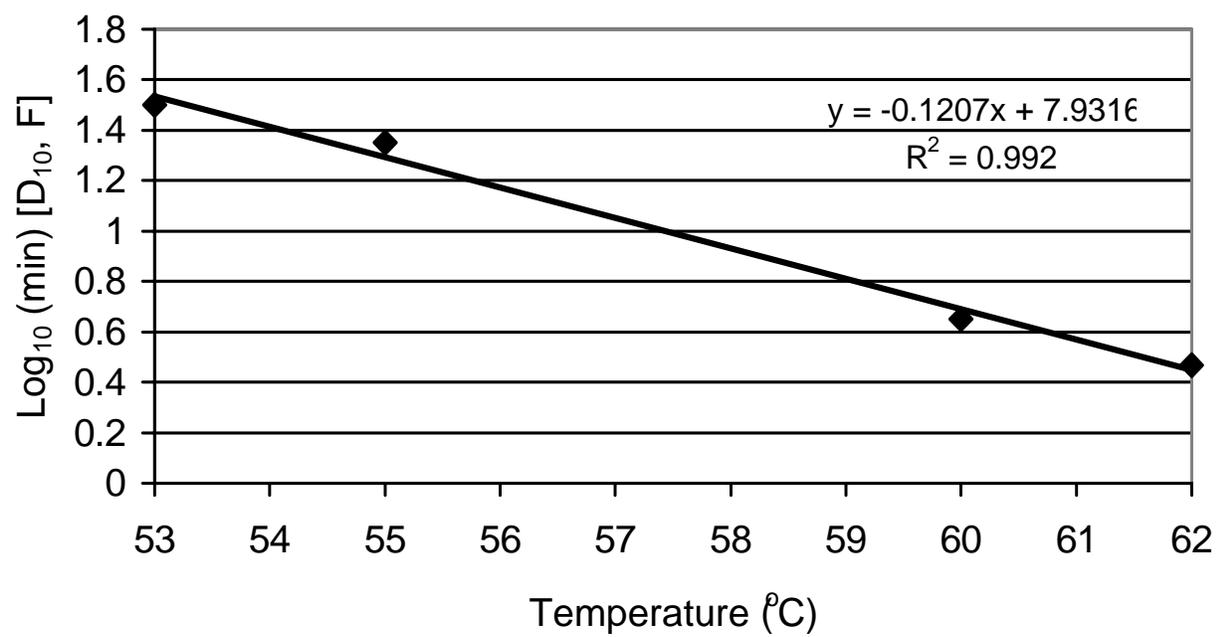


FIGURE 10. Thermal death time curve (7-D process) for *C. jejuni* on bologna surfaces (with trendline analysis equation).



**MANUSCRIPT 2**

**Inhibition of *Escherichia coli* O157:H7 and *Campylobacter jejuni* in Peptone Water  
and on the Surface of Turkey Bologna Using Nisin-Containing Antimicrobial Films and  
In-Package Pasteurization<sup>†</sup>**

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Key Words: *Escherichia coli* O157:H7, *Campylobacter jejuni*, Nisin, Antimicrobial Films,  
Inhibition

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† The use of trade names in this publication does not imply endorsement by the North  
Carolina Agricultural Research Service nor criticism of similar ones not mentioned.

## ABSTRACT

Nisin is an antimicrobial peptide (bacteriocin) produced by the food-grade microorganism *Lactococcus lactis* subsp. *Lactis*. This bacteriocin inhibits many Gram positive bacteria and when combined with chelating agents it inhibits some Gram negative organisms such as *Salmonella* and *E. coli* O157:H7. Because of nisin's GRAS (generally recognized as safe) status and its acceptance as a "natural" preservative, previous studies have been conducted to evaluate its potential to inhibit foodborne pathogens when incorporated into polymer and organic (edible) packaging films. The objective of this study was to examine the inhibitory activity of a wheat gluten (70% w/w) and glycerol (30% w/w)-based film containing 500 µg/ml nisin as Nisaplin, 3% citric acid, 5 mM EDTA, and 0.50% Tween 80 (Film+N) against *E. coli* O157:H7 and *C. jejuni* suspended in 0.1% peptone water. Alternatively, a control wheat gluten and glycerol-based film (Film) lacking nisin and the other treatment components and a 0.1% peptone water control (Control) were compared to the nisin-containing film. While the *E. coli* O157:H7 population increased 3-logs over the 72 hour exposure period (~23°C) for the Control and Film treatments, the Film+N treatment population decreased by 2-logs (a 5.6 to 7.4 log reduction). In contrast, *C. jejuni* populations decreased to below the minimum detectable level (log 1.2 cfu/ml) after 24 h following exposure to all three treatments. However, cells exposed to the Film+N treatment died at approximately twice the rate as the other two treatments.

The second objective of this project was to determine the inhibitory activity of these same film treatments following application to the surface of vacuum-packaged low fat turkey bologna samples that had been pre-inoculated with each bacterial test strain and then subjected to a minimal (< 1 log reduction) surface heat pasteurization process. Minimal

reductions in *E. coli* O157:H7 populations of 1.0, 0.5, and 0.35 log cfu/ml were detected across the Control, Film and Film+N treatments, respectively, following a seven day storage/exposure period. After an extended five week refrigerated storage period (Trial 3), the *E. coli* O157:H7 populations declined by 1.74, 1.14 and 0.27 log cfu/ml, respectively. For *C. jejuni*, population reductions of log 1.51, 2.11 and 2.68 cfu/ml were detected for the Control, Film and Film+N, respectively, following a 7-day refrigerated storage period. Cells exposed to the Film+N treatment died 1.5 to 2 times faster than cells exposed to the Control and Film treatments.

The findings of this study demonstrate that wheat gluten and glycerol packaging films containing nisin, chelators and a surfactant are generally more inhibitory against bacterial pathogens when suspended in a liquid system as opposed to contact with a relatively dry ready-to-eat food (bologna). It appears that at least for this film type and inhibitor, the inhibitory activity is limited by a lack of product moisture to facilitate migration and/or extraction of the treatment components from the film to the food surface. Further research is needed into how food systems and film types of different compositions and types as well as how environmental factors influence the efficacy of these inhibitory films. The ultimate goal is to develop a modified inhibitory packaging film that would prove useful as one of several food safety “hurdles” that collectively contribute to producing safer and higher quality food products having extended shelflives.

## INTRODUCTION

The United States has one of the safest food supplies in the world yet an estimated 76 million people become ill through foodborne agents each year (56). In 2001 there were 13,705 laboratory-diagnosed cases of foodborne diseases caused by the seven bacteria (*Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli* O157, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Vibrio* spp.) and two parasites (*Cryptosporidium* and *Cyclospora*) tracked under the FoodNet surveillance program. Of these nine foodborne disease agents, *Campylobacter* was ranked second (4,470 cases) to *Salmonella* (5,198 cases) as the most common cause of confirmed bacterial gastroenteritis in 2001. Although *E. coli* O157 accounted for only 4% of the foodborne diseases, the severity of this pathogen also warrants surveillance by the Centers for Disease Control and Prevention (CDCP) (14).

*C. jejuni* has been reported as the most common bacteria associated with human acute gastrointestinal infections in the U.S. (8), Canada (17, 36), Sweden (77), Scotland (11), and England (10). Symptoms of campylobacteriosis are generally seen two to five days after consumption of contaminated product. Infectious doses can be as low as 500-800 organisms (69) and the illness may persist up to 10 days (9, 13). *Campylobacter* spp. are thermophilic organisms with a growth range from 34° to 44°C and an optimal growth temperature of 42°C, which would reflect an adaptation to the intestines of warm-blooded birds (67). While the organism may not grow under refrigeration, it survives well in foods held at refrigeration temperatures (53). The organism will die more quickly in foods held at room temperature than when stored at 4°C (7, 25). For example, a 1- to 2-log reduction in *C. jejuni* was detected in chicken meat held for 17 days at 4°C, yet a 2.5- to 5-log reduction was observed in the same time frame when the chicken meat was held at 23°C (7).

*Escherichia coli* O157:H7 is of great concern to the food industry and regulatory agencies since this pathogen causes an estimated 20,000 cases of foodborne illnesses annually resulting in approximately 250 deaths and is the most common cause of hemolytic-uremic syndrome (HUS) in the U.S. (2). Since first identified as a pathogen, many different foods have been suspected or implicated as vehicles in outbreaks of *E. coli* O157:H7, with improperly cooked ground beef being linked epidemiologically to 40% of the disease outbreaks (15). Other meat products such as roast beef (70), salami (16), dry-cured salami (16), fermented salami (16, 79), and venison jerky (48) have also been implicated with disease outbreaks involving *E. coli* O157:H7. It is believed that there is a three- to four-day incubation period after ingestion of the *E. coli* O157:H7 organism. While most sufferers have a non-bloody diarrhea and abdominal cramps, patients that seek medical attention usually have bloody diarrhea that is typically observed during the second or third day of the illness (63). The illness generally lasts for one week, yet 6% of the patients develop hemolytic-uremic syndrome (HUS) (35). The infectious dose is believed to be relatively low. One investigation estimated the infectious dose to be less than 50 organisms, with some of the cases having an estimated infectious dose of less than five organisms (79). Studies have shown that *E. coli* strains which produce Shiga toxins (STEC) were present in 15-40% of the ground beef samples tested in Canada (45), 23-25% in the U.S. (1, 71), 17% in the United Kingdom (85), and 16.1% in the Netherlands (38). In another study, 3.7% (6/164) of beef samples, 1.5% (4/263) of poultry samples, and 2% (4/200) of lamb samples tested positive for *E. coli* O157:H7 (26).

Over the past thirty years the per capita consumption of chicken has more than doubled in the U.S., reaching 54 lb (24.5 Kg) in 1999 (81). The poultry industry began

marketing more cut-up and further processed products in the 1950's and 60's which included the use of mechanically separated meat from carcass parts such as frames, necks, and backs. Mechanically separated poultry meat is used in producing such emulsified products as bologna, salami, and frankfurters (33). These ready-to-eat (RTE) products undergo a cooking process that is designed to eliminate all pathogenic bacteria, chilled to  $\leq 40^{\circ}\text{F}$  ( $\leq 4.4^{\circ}\text{C}$ ), and then stored in coolers at 26 to 28 $^{\circ}\text{F}$  (-3.3 to -2.2 $^{\circ}\text{C}$ ) before the casing is mechanically peeled from frankfurters or sliced in the case of bologna (49). The equipment used in further processing plants such as slicers, dicers, and brining and packaging machines have been shown to harbor pathogenic bacteria, namely *Listeria monocytogenes* (4, 57, 80). Studies have documented that the bacterial loads in commercially produced RTE meat products can increase from  $\log_{10}$  0.5 to 2.0 cfu/g of product during the slicing process alone (39). Post-process handling is considered to be the primary cause of contamination of RTE meat and poultry products (18, 83).

A major goal of federal and state regulatory and public health agencies and food manufacturers is the prevention of human illnesses caused by foodborne pathogens. This goal has been partially achieved by the introduction of HACCP-based food safety principles in many U.S. and foreign food processing plants (82). However, other innovative approaches for improving food safety are needed such as the development of "active" packaging systems to reduce foodborne pathogen populations residing on the surface of packaged products. Active packaging provides more than just an inert barrier between the product and the external environment (40). For example, in one type of active packaging, antimicrobial agents have been incorporated directly into polymeric or edible packaging materials. This innovation allows the food industry to combine the merits of existing packaging materials

with the preservative functions of antimicrobials (62, 84). To gain regulatory approval for such food package applications generally requires extensive time and money. A more expedient route of gaining approval of these applications may be through the use of “naturally” occurring antimicrobials such as the inhibitory peptide nisin (a bacteriocin). This approach may be defined as “biopreservation” of foods which involves inhibiting or destroying undesirable microorganisms in foods through the use of antagonistic microorganisms (76) or their metabolites (58). This type of approach has been examined by a number of investigators (27, 28, 50, 51, 52, 59, 60, 61, 68, 72).

Nisin is produced during the exponential growth phase by the lactic-acid producing bacteria *L. lactis* subsp. *lactis*, a common microorganism used to ferment dairy food products (12, 43, 44). A joint FAO/WHO expert committee recognized nisin as a safe and legal biological food preservative in 1969 (30). Since then, nisin received GRAS (generally recognized as safe) status in the United States (31) for use in several food applications including some pasteurized cheese spreads to prevent spore outgrowth and toxin production by *Clostridium botulinum* (23). Studies have demonstrated that nisin has a wide range of inhibitory activity against Gram positive bacteria (42, 46) and some Gram negative bacteria that have been pre-sensitized by exposure to cell membrane-permeabilizing treatments (75, 64). For example, alteration of the outer cell membrane of *E. coli* by physical treatments such as osmotic shock (54) and ultra high pressure (47, 73) resulted in an increased sensitivity of this organism to nisin. The standard approach to reducing nisin resistance of Gram negative bacteria has been exposure to metal chelators (6, 20, 29, 75). Chelating agents (i.e., EDTA and citric acid) increase the permeability of the outer bacterial membrane by binding divalent cations (i.e.  $Mg^{2+}$ ,  $Ca^{2+}$ ) located in the lipopolysaccharide (LPS) layer

of the outer membrane (6, 75). The loss of cations in the LPS region destabilizes the outer membrane leading to an increase in nisin migration to the cytoplasmic membrane, the ultimate site of nisin's inhibitory activity (75). This approach may lead to less severe heat treatments if the heating step is preceded by the addition of bacteriocins (5, 32, 37). By reducing the heating time, some food products are anticipated to have improved quality and nutritional value, not to mention lower processing costs and longer run times between cleaning (34).

The objectives of this study were to determine if wheat gluten films containing nisin, citric acid, EDTA and Tween 80 effectively inhibit *C. jejuni* and *E. coli* O157:H7. The initial objective of this study was to determine the efficacy of these films against a suspension of each organism in 0.1% peptone water. The second objective was designed to determine the efficacy of a minimal surface heat pasteurization treatment combined with antimicrobial films on inhibiting *C. jejuni* and *E. coli* O157:H7 on the surface of vacuum packaged, low fat turkey bologna. This study is part of a comprehensive study with colleagues from Clemson University who conducted similar studies with *Listeria monocytogenes* and *Salmonella* Typhimurium.

## MATERIALS AND METHODS

**Bacterial cultures.** The *E. coli* O157:H7 culture strains used in this study (and their sources) included HC 1840 (human isolate), HC 19386 (human isolate), and HC 766 (human isolate) obtained from the Bureau of Microbial Hazards, Health and Welfare Canada, Ottawa, Ontario, Canada; and ATCC 43894 (human isolate) obtained from the American Type Culture Collection (Rockville, MD). *Campylobacter jejuni* strains WCR 72927, WCR

71940, and EPI 36 (environmental strains isolated from poultry production and processing facilities) were obtained from Dr. Eric Line, USDA-ARS (Athens, GA).

**Culture maintenance and inoculum preparation.** Stock cultures of *E. coli* O157:H7 strains were maintained in sterile double strength brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) and glycerol (50:50 vol.) and stored at -20°C. A 0.1% (vol/vol) inoculum of each stock culture was used to prepare a fresh BHI culture (incubation at 37°C for 24 h with agitation). For each *E. coli* O157:H7 strain, 10- $\mu$ l samples of culture were transferred to 10 ml of fresh BHI broth and incubated at 37°C for 24 h with agitation. The 10-ml cultures were pooled together (four strains) and centrifuged at 12,000 x g for 12 minutes at 5°C. The pellet was washed twice in 0.1% peptone water, centrifuged again as previously described, and resuspended in a volume of 0.1% peptone water to obtain a targeted population of approximately log<sub>10</sub> 4.2 cfu/ml for the peptone water study and log<sub>10</sub> 9.0 cfu/ml for the bologna study.

*Campylobacter jejuni* strains were maintained on Campy Cefex agar (Gibson Laboratory, Lexington, KY) at 4°C and then transferred to Brucella broth (Difco Laboratories, Detroit, MI) 4-days prior to initiating an experiment (42°C for 48 h in a microaerophilic gas environment of 5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>). Thirty  $\mu$ l of each test culture (0.1% inoculum, vol/vol) were transferred to 30 ml of fresh Brucella broth and incubated as previously described. The 30 ml cultures were pooled together, centrifuged, washed twice in 0.1% peptone water, and resuspended in 0.1% peptone water according to the procedures described for *E. coli* O157:H7. The targeted population for the peptone water study was approximately log<sub>10</sub> 5.2 cfu/ml and log<sub>10</sub> 8.5 cfu/ml for the bologna study.

**Bologna.** A low fat turkey bologna averaging 66.1% water (102°C for 18h), 13.8% fat (Soxhlet), 11.0% protein (Kjeldahl), 6.45% carbohydrate (by difference), and 2.71% ash (525°C for 24h) was obtained from a commercial source, cut into 3.84 by 3.84 cm samples, packaged in Cryovac P64x pouches (Cryovac Inc., Duncan, SC), and frozen before being irradiated with a 10kGy dose for 79 minutes using a Cobalt 60 source (Auburn University, AL) (3). Samples were irradiated to reduce the background microflora but not to necessarily produce sterility. The background microflora populations following irradiation ranged from 1.5 to 13.5 cfu/gm of bologna. Irradiated bologna samples were stored at -20°C and thawed for 24 h at 4°C prior to initiating an experiment.

**Reagents.** Nisaplin® (Aplin and Barrett, Dorsett England) was stored at -20°C. The chelating agents and surfactants used in this study were ethylenediaminetetraacetate (EDTA) (Fisher Scientific Co., Dallas, TX), citric acid anhydrous (citrate) (Sigma Chemical Co., St. Louis, MO), and Polyoxyethylenesorbitan (Tween 80) (Sigma Chemical Co., St. Louis, MO).

**Film and treatment formulations.** The experimental films were composed of a 7:3 ratio (w/w) of wheat gluten (Sigma Chemical Co., St. Louis, MO) and glycerol (Fisher Biotech, Fair Lawn, NJ) (78) and formed using a heat-press technique according to Dawson *et al.*, (22). The nisin-containing film (Film+N) was composed of 7% Nisaplin® (w/w) (i.e., 2.5% nisin with NaCl and denatured milk solids), 3% citric acid, 5 mM EDTA, and 0.50% Tween 80 (all w/w basis) whereas the control film (Film) contained only wheat gluten and glycerol. The film was formed by placing 0.9 g of the protein, glycerol and other component mixture onto a 3.84 by 3.84 cm aluminum template. The template was then pressed at  $1.38^8$  Pa (20,000 psi) for 1.5 to 3 minutes between heated (140°C, 284°F) aluminum plates (Tetrahedron Associates, San Diego, CA). Nisin-containing films were

composed of 63 mg of Nisaplin or 1.58 mg of pure nisin per 3.84 by 3.84 cm film (60,040 IU of pure nisin/film piece). A final mean film thickness of 65.5  $\mu\text{m}$  was determined by taking five different measurements per sample using a hand-held micrometer (AMES, Waltham, MA). After air cooling under a laminar flow hood for 5 minutes at ambient temperatures ( $\sim 23^{\circ}\text{C}$ ), the film samples were aseptically removed from the template, placed in petri dishes (Fisher Scientific, Fair Lawn, NJ), sealed with parafilm (American National Can, Greenwich, CT), and stored at  $4^{\circ}\text{C}$  in Ziplock bags (S.C. Johnson & Son, Inc., Racine, WI) to prevent desiccation of films.

**Standard protocol for inactivation in peptone water.** Thirty ml of each test organism in 0.1% peptone water were aseptically transferred into triplicate 75ml Nunc EasY Vented Flasks (Fisher Scientific, Fair Lawn, NJ) (per treatment) containing one 3.84 by 3.84 cm Film+N, Film, or Control (no film) treatments. Flasks containing the *C. jejuni* suspensions were subsequently placed in freezer bags (Ziploc, S.C. Johnson and Son Inc., Racine, WI), flushed with a microaerophilic environment (5%  $\text{O}_2$ , 10%  $\text{CO}_2$  and 85%  $\text{N}_2$ ) and then sealed. All flasks were placed on a Lab-line Orbit Environ-Shaker (Lab-line Instruments Inc., Melrose Park, IL) and agitated (50 RPM) at room temperature (approximately  $23^{\circ}\text{C}$ ). *C. jejuni* samples (0.1 and/or 1.0 ml) were aseptically taken at 0, 2, 4, 8, 24 and 48 hours, serially diluted in 0.1% peptone water prior to being surface plated (0.1 ml) on Brucella agar (Gibson Laboratories, Lexington, KY), and incubated at  $42^{\circ}\text{C}$  for 48 h in a microaerophilic gas environment as previously described. *E. coli* O157:H7 samples were aseptically taken at 0, 2, 4, 8, 24, 48 and 72 hours, serially diluted in 0.1% peptone water, surface plated (0.1 ml) onto BHI agar, and incubated for 48 hours at  $37^{\circ}\text{C}$ . Colonies

were subsequently enumerated and the populations transformed to  $\log_{10}$  cfu/ml of diluent.

Experiments were run in triplicate using fresh cultures and triplicate samples per treatment.

**Standard protocol for inactivation by heat and films.** Twenty five microliters of resuspended cells of each pooled organism in 0.1% peptone were aseptically spread across the top surface of each irradiated bologna sample using a sterile L-shaped glass rod and air dried for 15 minutes. Target populations were  $\log$  7.4 and 6.9 cfu/sample for *E. coli* O157:H7 and *C. jejuni*, respectively. The appropriate film treatment (Control, Film, Film+N) was aseptically applied to the top surface of triplicate pieces of bologna and then transferred to a 22 cm x 15.3 cm, 2.6 mil multilaminate pouch composed of nylon skin and barrier layers and a linear low-density polyethylene sealant layer (P64X pouch, oxygen transmission rate of 60cc/m<sup>2</sup>/day at 23°C and 0% relative humidity, Cryovac Inc., Duncan, SC). Each pouch was vacuumed sealed with one single layer of product in three separate sealed chambers per pouch. The sealed pouches were immersed in a preheated circulating water bath (60°C for 60 sec) equipped with a calibrated temperature control module accurate to  $\pm 0.05^\circ\text{C}$  (Model DC1, Haake Inc., Karlsruhe, Germany). After 60 sec, the samples were removed from the water bath and rapidly cooled for 10 min in an ice-water slurry. Sample surface temperatures were monitored over the course of the heating and cooling cycles (1 second intervals) using type K thermocouples attached to a Tempest Datalogger (Tangent Systems, Inc., Charlotte, NC).

Bologna samples were then stored at 4°C for up to 7 days with one pouch containing three pieces of bologna per treatment sampled at 24 hour increments. The three bologna samples were aseptically removed from each pouch and placed into separate stomacher filter bags (Spiral Tech, Norwood, Massachusetts) containing 5 or 10 ml of 0.1% peptone water

and then blended for one minute using an IUL Masticator (IUC Instruments, Cincinnati, OH). This initial dilution was then serially diluted in 0.1% peptone water and surface-plated onto Sorbital MacConkey Agar for *E. coli* O157:H7 (37°C for 24 h, Gibson Laboratory, Lexington, KY) or Campy Cefex agar for *C. jejuni* (42°C for 48 h, Gibson Laboratory, Lexington, KY). Campy Cefex plates were placed in freezer bags (Ziploc, S.C. Johnson and Son Inc., Racine, WI) and then incubated for 48h in a microaerophilic environment as previously described. Following incubation, colonies were enumerated for each treatment and sampling time.

Following the completion of the seven day storage study, an additional storage study extending out to five weeks was conducted using only *E. coli* O157:H7. The procedures were identical as discussed above except samples were taken on a weekly basis for 5 weeks.

**Statistical analysis.** Data collected from each experiment were analyzed using the general linear model (GLM) statistical design of SAS 2000 (Version 8.0, Statistical Analysis System, Cary, NC). Replication, film treatment, exposure time, and treatment by time interactions were included in the GLM model with the residual interaction effects comprising the error term. Where the main effects were significant ( $P \leq 0.05$ ), the means were separated using least square means. Regression analyses were also run to determine if effects were quadratic or linear.

## RESULTS AND DISCUSSION

**Raw product composition.** The proximate composition of the low fat turkey bologna averaged 66.1% moisture, 11.0% protein (w/w, wet basis) 13.8% fat (w/w, wet basis), and 2.7% ash (w/w, wet basis).

**Inactivation in peptone water study.** The impact of the three treatments (Control, Film, Film+N) on *E. coli* O157:H7 and *C. jejuni* populations suspended in 0.1% peptone water is summarized in Figures 1 and 2, respectively. The initial *E. coli* O157:H7 population averaged log 4.2 cfu/ml. Linear increases ( $r = 0.90$  and  $0.88$ , respectively) in the *E. coli* O157:H7 populations were observed after the fourth hour of incubation at room temperature ( $\sim 23^{\circ}\text{C}$ ) for both the Control and Film treatments. Populations in both treatments reached a maximum of 7.21 and 8.95 log cfu/ml, respectively, after 48 and 72 hours. The linear regression slopes for the linear portion of each plot were 0.054 and 0.058, respectively. Compared to the Control treatment (cells suspended in 0.1% peptone water), the Films containing wheat gluten and glycerol appeared to promote the growth of the *E. coli* test strains more so than the Control treatment. This was especially evident over the final 48 hours of incubation. In contrast, the film containing nisin and the other treatment components (chelators, acidulant, surfactant; Film+N) inhibited the four-strain *E. coli* O157:H7 cocktail. After 4 hours of exposure, cells exposed to the nisin-containing films began a linear ( $R^2 = 0.961$ ) decline in population [decimal reduction time ( $D_{23^{\circ}\text{C}}$ ) = 27.8 hours] that reached log 2.19 cfu/ml after 72 hours (a 2-log reduction). Compared to the Film and Control treatments, the Film+N treatment yielded an average reduction in cell population of 6.76 and 4.96 logs, respectively, after 72 hours. These findings indicate that nisin and the other treatment components co-migrated from the film into the peptone water to inhibit the *E. coli* O157:H7 test strains. Results of the statistical analyses indicated significant time, treatment, and time by treatment interactions ( $P = 0.0001$ ). The least squares treatment means for the Control, Film, and Film+N treatments were log 5.62, 6.34, and 3.39 cfu/ml,

respectively ( $P < 0.0001$ ). Each least squares means was significantly different from the other two least squares means.

In a previous study by Stevens *et al.* (75), they detected a 3.2 to 6.9-log reduction in population for 20 *Salmonella* serotypes and an *E. coli* O157:H7 human isolate following a 1-hour exposure to 50 µg/ml of nisin and 20 mM disodium EDTA in a Tris buffer system. In contrast, when each component was tested separately, no significant inhibition (< 1-log cycle reduction) of the test species was observed. They hypothesized that the population reductions by nisin are facilitated by the chelation of magnesium ions, present in the outer membrane, by EDTA. The removal of magnesium ions from the lipopolysaccharide (LPS) layer of the outer membrane results in loss of LPS and an increase in cell permeability. This increase in outer membrane permeability to nisin is proposed to facilitate inactivation of the cell via bactericidal action at the cytoplasmic membrane.

In view of the Stevens *et al.*, (75) study, the findings from the present study indicate that both nisin and the other treatment components must have co-migrated or were co-extracted from the film into the peptone water to produce this level of inhibition. However, the level of inhibition achieved over the 72 hour exposure period was significantly less (~2 log cycles) than the 6.9 log reduction achieved by Stevens *et al.* (75) following only a 1-hour exposure period in Tris buffer. Although the initial concentration of nisin was significantly higher in the present study (1580 µg versus 50 µg for the Stevens study), the fact that nisin and the other treatment components were contained within the wheat gluten protein matrix may explain the reduced level of inhibition. A similar finding was observed in a study by Natrajan and Sheldon (60) where 500µg/ml of nisin was added to an edible film composed of agar concentrations of 0.75% and 1.25% and subsequently applied to broiler drumstick skin

inoculated with a *Salmonella* Typhimurium test strain. While the level of inhibition of *Salmonella* Typhimurium was not significantly different between the two film treatments at 24, 48 and 72 hours of exposure, skin exposed for 96 hours to the 0.75% agar film had significantly lower *S. Typhimurium* populations than broiler skin treated with the 1.25% agar film. They theorized that the degree of cross-linking of the agar film as related to agar concentration may have influenced the rate and degree of migration of nisin formulation components onto the surface of the broiler drumstick skin (60).

For *C. jejuni*, the initial populations averaged log 5.23 cfu/ml of diluent. In contrast to *E. coli* O157:H7, *C. jejuni* populations decreased across all three treatments. Linear decreases ( $r = 0.91, 0.83$  and  $0.96$ , respectively) in the *C. jejuni* population were detected throughout the sampling periods across the Control, Film and Film+N treatments. By 24 hours all treatments had reached the minimal detection level of log 1.2 cfu/ml. The three treatment D-values derived from the slopes of the linear regression equations were 7.2, 6.8, and 3.2 hours, respectively for the Control, Film and Film+Nisin treatments. As noted, the rates of inactivation were similar for the Control and Film treatments. Compared to the initial inoculum population of 5.23 log cfu/ml, the 12-hour treatment populations averaged log 2.64, 1.51, and 1.20 cfu/ml, a 2.59, 3.72, and  $\geq 4.03$  log reduction in population, respectively. Moreover, the flasks containing the two film treatments had 12-hour populations of 1.13 and  $\geq 1.44$  log cfu/ml lower than the Control treatment. The ANOVA GLM procedure identified significant differences between sample times ( $P = 0.0001$ ) but not for treatments ( $P = 0.099$ ) or treatment by time interactions ( $P = 0.32$ , see Table 1). The decline in population may be due in part to the fastidiousness of the organism. Although the flasks containing the inoculum were maintained under a microaerophilic environment, the

redox potential, temperature, and nutrient requirements for this organism may not have been optimal for growth and survival. For example, previous studies have shown that *C. jejuni* dies more rapidly in foods held at room temperature than when stored at 4°C (7, 25). In the first study, the investigators recorded a 1- to 2-log reduction in *C. jejuni* population in chicken meat held for 17 days at 4°C, yet a 2.5- to 5-log reduction when held at 23°C (7).

**Heat and film inactivation study.** Although the above study demonstrated that when wheat gluten-based films containing nisin and other treatment components are suspended in 0.1% peptone water they are effective in inhibiting *E. coli* O157:H7 and to a lesser degree *C. jejuni*. However, additional studies were warranted to determine the effectiveness of these films after application to ready-to-eat food products. Previous studies have documented that the inhibitory activity of bacteriocins against foodborne pathogens is greatly influenced by several intrinsic factors of the food including pH, lipid content, presence of proteolytic enzymes, and liquid versus solid systems (66). Thus, the efficacy of these active packaging systems in controlling pathogens on food surfaces may be severely limited based on these and other factors.

A typical sample temperature profile of the bologna surface undergoing a surface heat pasteurization process is presented in Figure 3. The basis for subjecting the inoculated bologna to an initial heat pasteurization step was to first effect a minimal (<1 log) reduction in bacterial population but more importantly to injure or sensitize the cells such that they are more susceptible to nisin inactivation. The average heating come-up time for the surface of the bologna to reach the targeted 60°C was around 46 seconds where it remained for an additional 14 seconds followed by cooling in an ice-water slurry (~40 seconds to reach 4°C).

Based on preliminary results (41), it was determined that a heating time of this duration would yield a reduction in bacterial population of around 1 log.

The results of the combined heat and film treatments on *E. coli* O157:H7 and *C. jejuni* populations inoculated on bologna are summarized in Figures 4 and 5, respectively. The initial *E. coli* O157:H7 population averaged log 7.87 cfu/ml prior to heating. Following 60 seconds at 60°C, the population declined about 0.5 log to 7.31, 7.28 and 7.38 for the Control, Film and Film+N treatments, respectively. The Control treatment population gradually decreased over the seven-day sampling period reaching a final population of log 6.31 cfu/ml (a 1.0 log reduction). The Film treatment population declined to log 6.79 cfu/ml by day 7 (0.5 log reduction). Similarly, the Film+N treatment population declined to log 7.03 cfu/ml by day 7 (0.35 log reduction). No significant treatment or treatment by time interactions were observed although significant time effects were detected. Moreover, when D-values were calculated from the slope of the linear portion of each treatment survivor curve, there were some notable differences between the three treatments (Control – 7.2 days; Film – 8.5 days; Film+N – 6.1 days). Cells exposed to the films containing nisin died at a faster rate than those exposed to the control wheat gluten films (1.4 times faster) or to no films (1.2 times faster). These findings clearly demonstrated some minimal inhibitory activity attributed to the nisin-treated films. Unlike the initial trials conducted in peptone water, this lack of inhibition indicates a reduction in migration of nisin and/or other treatment components from the film to the bologna surface. Since the moisture content of bologna is relatively low (66%), there would be minimal free water available in or on the surface of the product to aid in the extraction and migration of the treatment components from the films. Natrajan and Sheldon (60) arrived at a similar conclusion in a study they conducted to

evaluate the efficacy of nisin-coated polymer films (nylon, linear low density polyethylene, polyvinyl chloride) to inactivate *Salmonella* Typhimurium inoculated on the surface of fresh broiler drumstick skin. Skin samples that had been pre-moistened with water yielded significantly greater inhibition than dry skin samples. They attributed the lack of inhibition to a lack of available product surface moisture to aid in solubilizing the treatment components. A second possibility is that one or more treatment components may not co-migrate from the films at the same rate. If this latter scenario was the case, nisin inhibitory activity against both organisms would be lost or greatly reduced. Previous studies by Stevens *et al.*, (75) demonstrated that nisin was not inhibitory against *Salmonella* species and other Gram negative bacteria when cells were sequentially treated; first with EDTA then followed by nisin. Conversely, cells treated simultaneously with nisin and EDTA exhibited a significant reduction in population of 3 to 7 logs.

The initial population of *C. jejuni* inoculated on the surface of the bologna averaged log 6.48 cfu/ml. After the same preliminary heat treatment of 60°C for 60 seconds, the populations decreased to 4.72, 5.82, and 5.20 for the Control, Film and Film+N, respectively. A gradual decrease in population was observed across all three treatments reaching a 7-day final population of log 3.21, 3.71 and 2.52 cfu/ml, respectively. Compared to the initial population, these 7-day populations represent a 1.51, 2.11, 2.68 log reduction for each treatment, respectively. The corresponding treatment D-values calculated from the linear portion of the survivor curves were 2.6, 3.4, and 1.7 days for the Control, Film, and Film+N treatments, respectively. *Campylobacter jejuni* populations exposed to the films containing nisin died approximately 1.5 times faster than the control treatment and 2 times faster than cells exposed to the untreated films. Significant treatment ( $P < 0.0001$ ) and time ( $P <$

0.0001) effects were detected. The least square means for the Control, Film, and Film+N were log 4.19, 4.42 and 3.06 cfu/ml, respectively ( $P < 0.0001$ ). These findings demonstrate that the Film+N treatment had a greater impact on decreasing the growth of the microorganism over time compared to the other two treatments. The least square mean for the Film treatment was slightly higher (0.23 log) than the Control indicating that the presence of the film may actually support the growth of the microorganism. Another possibility may be related to the protective effect of the film in preventing desiccation of the bologna surface and *Campylobacter* which is known to be highly sensitive to dehydration. The films may have also provided some protective insulation effect for the organism during the heat treatment.

In order to determine whether a longer exposure time to the nisin-containing film would result in greater inhibition of *E. coli* O157:H7, a final study was conducted which involved refrigerating the treated bologna samples for five weeks. The resulting survivor populations are summarized in Figure 6. The initial *E. coli* O157:H7 population averaged log 7.63 prior to the heat treatment (60°C for 60 seconds) and log 6.88 cfu/ml following heating (0.75 log reduction). A gradual but insignificant decrease in population was observed with the Film+N treatment, reaching a minimum population of 6.1 log cfu/ml at week 4 followed by an increase to log 6.6 cfu/ml by week 5. A similar survivor profile was observed for the Film treatment where at 5 weeks the population had declined to log 5.74 cfu/ml (a 1.1 log reduction). The greatest decline in population was observed in the Control treatment where a 1.74 log reduction was recorded after week 5. The statistical analysis indicated significant treatment ( $P < 0.0001$ ) and time ( $P < 0.0001$ ) effects but no significant time by treatment ( $P = 0.1136$ ) interactions. The Least Squares means for the three

treatments were 5.81, 6.19, and 6.63, respectively ( $P < 0.0001$ ). Each Least Squares mean was significantly different from the other two treatments Least Squares means. These findings further indicate that incorporation of nisin and the other treatment components into the wheat gluten and glycerol films may actually interfere with the nisin inhibition process by retarding or preventing migration of the components. Moreover, wheat gluten may also provide nutrients for the organisms as noted by higher surviving populations on samples containing the control or treated films.

Natrajan and Sheldon (61) noted similar findings in a study to determine the extent of inhibition of *S. Typhimurium* on chicken skin coated with a polysaccharide- (calcium alginate) and protein-based (agar) film containing varying concentrations of nisin and 5 mM EDTA, 3% citric acid, and 0.5% Tween 80. Films containing nisin, chelators, and surfactant resulted in cell population reductions ranging from 2 to 3 logs and 1.8 to 4.6 logs, respectively, for the polysaccharide- and protein-based films. They observed that the lower concentration of agar (0.75% by wt) yielded significantly larger population reductions (4.6-log) than the 1.25% agar film, presumably due to the greater protein cross-linking in the higher protein-containing film. They hypothesized that the more highly cross-linked protein film had a more closed and rigid structure that retarded migration of nisin and the other treatment components. Moreover, they further noted that for bacterial inhibition to occur from contact with these films, nisin and the chelators would need to migrate from the film at a similar rate in order for nisin to inhibit *Salmonella*. Experiments conducted by Stevens *et al.* (75) demonstrated that the inhibition of *S. Typhimurium* with nisin and EDTA is mediated by the ability of EDTA or other chelators (i.e., citric acid) to bind calcium and magnesium divalent cations located in the LPS region of the outer membrane resulting in a

destabilization of the membrane and increase in permeability. Nisin subsequently migrates to the inner cytoplasmic membrane where it alters the membrane's surface free energy leading to a change in permeability and ultimately cell lysis (55). In the present study we speculated that suspending the treated films in the peptone water actually aided in the extraction of nisin and other treatment components into the culture suspension leading to bacterial inhibition.

Other factors that may have contributed to a loss in inhibition from the Film+N treatment when applied to bologna is the hydrophobic nature of nisin which causes it to be more attracted to lipids in foods. This attraction with hydrophobic molecules may lead to an heterogeneous distribution in foods leading to a loss in antibacterial activity (65). Moreover, studies by Cutter and Siragusa demonstrated that nisin and nisin with EDTA are less effective against Gram negative bacteria when the organisms are attached to beef tissue surfaces than when cells are suspended in broth (19, 20, 21). They proposed five possible reasons for this occurrence. The first factor is that nisin may have been degraded by endogenous proteases present in the meat. Second, nisin may preferentially bind to adipose tissue resulting in a heterogeneous distribution of nisin across the tissues. Third, the solubility of nisin may decrease at the near neutral pH of meats. Fourth, the presence of other ions on meat surfaces may interfere with the binding of EDTA to magnesium in the bacterial outer membrane lipopolysaccharide layer. The fifth explanation is that nisin can readily bind to meat proteins reducing their availability to bind to bacterial cells (20, 24). However, in a study by Stevens *et al.* (74), the effects of an added protein on nisin activity proved otherwise. In their study they examined the effects of adding bovine serum albumin in a pH 6.5 Tris buffer system on nisin activity. The treatments included adding 1mg/ml of

BSA to each of four treatments: (i) cell buffer, (ii), 20 mM EDTA in cell buffer, (iii) 50 µg/ml nisin in cell buffer and (iv) 20 mM EDTA and 50 µg/ml nisin in cell buffer. Although the added protein was 20 times the concentration of nisin, no reduction in nisin/EDTA activity was detected against six *Salmonella* serotypes. Although it is anticipated that all endogenous meat proteases were heat inactivated during the cooking process, the other four explanations may have influenced the outcome of this objective.

For these reasons and possibly others, before antimicrobial films are used in food packaging processes, their effectiveness against pathogenic bacteria should be tested in different food systems. While active packaging materials may not completely inhibit pathogenic microorganisms by themselves, they may be used as an additional safety “hurdle” step in food processing plants having HACCP programs. Another possible means for enhancing the bacteriocidal activity of nisin in wheat gluten films is to increase the surface heat pasteurization temperatures or process times for treating the bologna so as to increase the sensitivity of the bacterial pathogens to nisin. Previous studies have shown that some Gram negative bacteria are sensitized to nisin after being exposed to treatments that change the permeability of their outer membrane (64, 75). For example, alteration of the outer membrane of *E. coli* by physical treatments such as osmotic shock (54) or ultra high pressure (47, 73) increased the sensitivity of this organism to nisin. Moreover, treatment with chelators such as EDTA was shown to increase the sensitivity of *E. coli* O157:H7 to nisin (20, 29).

Although the findings of objective 1 (both organisms) and objective 2 (*C. jejuni*) were somewhat encouraging, the lack of consistent inhibition under objective 2 justifies the need for further research into how food systems and film types of different compositions and types

as well as environmental factors influence the efficacy of these inhibitory films. The ultimate goal would be to develop a modified inhibitory packaging film that would prove useful as one of several food safety “hurdles” that collectively contribute to producing safer and higher quality food products having extended shelf lives.

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TABLE 1. ANOVA table of objectives 1 (films suspended in peptone water) and 2 (films applied to a low fat turkey bologna and then subjected to a surface heat pasteurization process).

<b>Objective</b>	<b>Bacteria</b>	<b>Source</b>	<b>F Value</b>	<b>Pr &gt; F</b>
<b>Objective 1</b>	<i>E. coli</i> O157:H7	Treatment	473.97	<0.0001
		Time	51.00	<0.0001
		Treatment*Time	51.00	<0.0001
<b>Objective 1</b>	<i>C. jejuni</i>	Treatment	2.62	0.0986
		Time	23.38	<0.0001
		Treatment*Time	1.25	0.3253
<b>Objective 2</b>	<i>E. coli</i> O157:H7	Treatment	5.78	0.0058
		Time	9.60	<0.0001
		Treatment*Time	0.85	0.6151
<b>Objective 2</b>	<i>C. jejuni</i>	Treatment	32.60	<0.0001
		Time	17.29	<0.0001
		Treatment*Time	1.63	0.1136

FIGURE 1. Inhibitory effect of wheat gluten and glycerol films containing nisin, chelators, and a surfactant on populations of *E. coli* O157:H7 suspended in 0.1% peptone water (Control – no film; Film – film without treatment components; Film+N – films containing nisin and other treatment components; with linear regression equations).

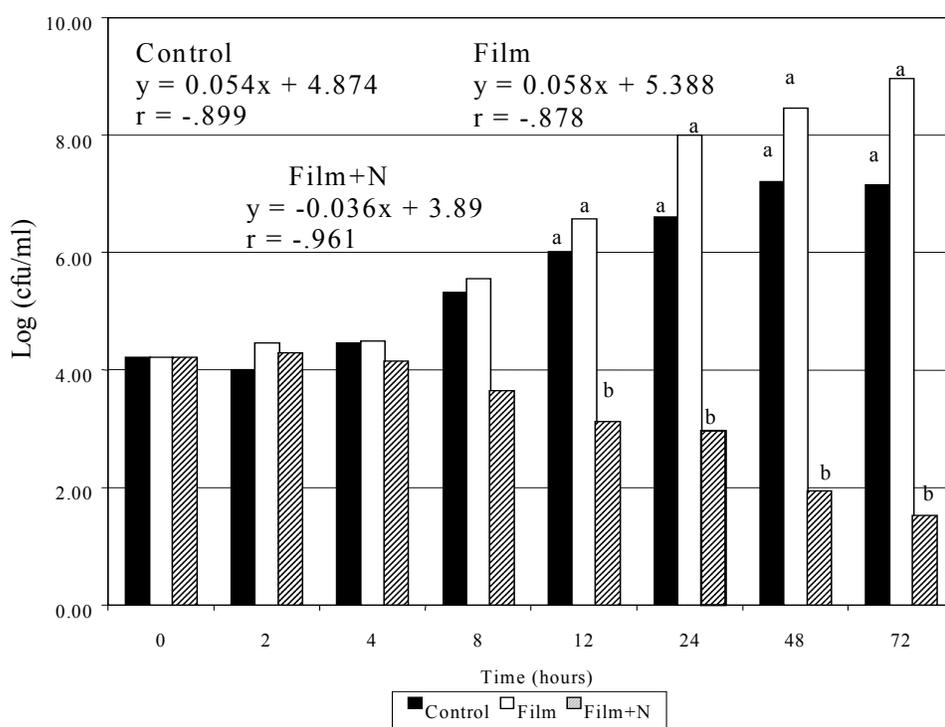


FIGURE 2. Inhibitory effect of wheat gluten and glycerol films containing nisin, chelators, and a surfactant on populations of *C. jejuni* suspended in 0.1% peptone water (Control – no film; Film – film without treatment components; Film+N – films containing nisin and other treatment components; with linear regression equations).

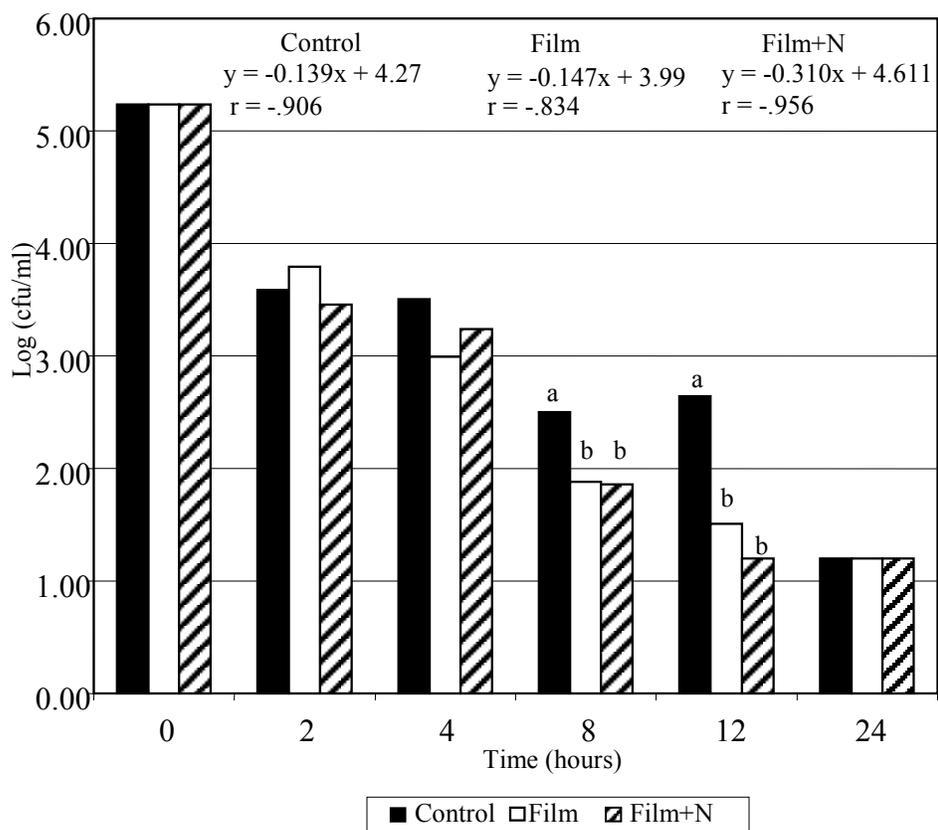


FIGURE 3. Typical surface temperature heating profile of a single layer of low fat turkey bologna vacuum packaged in a multilaminate pouch.

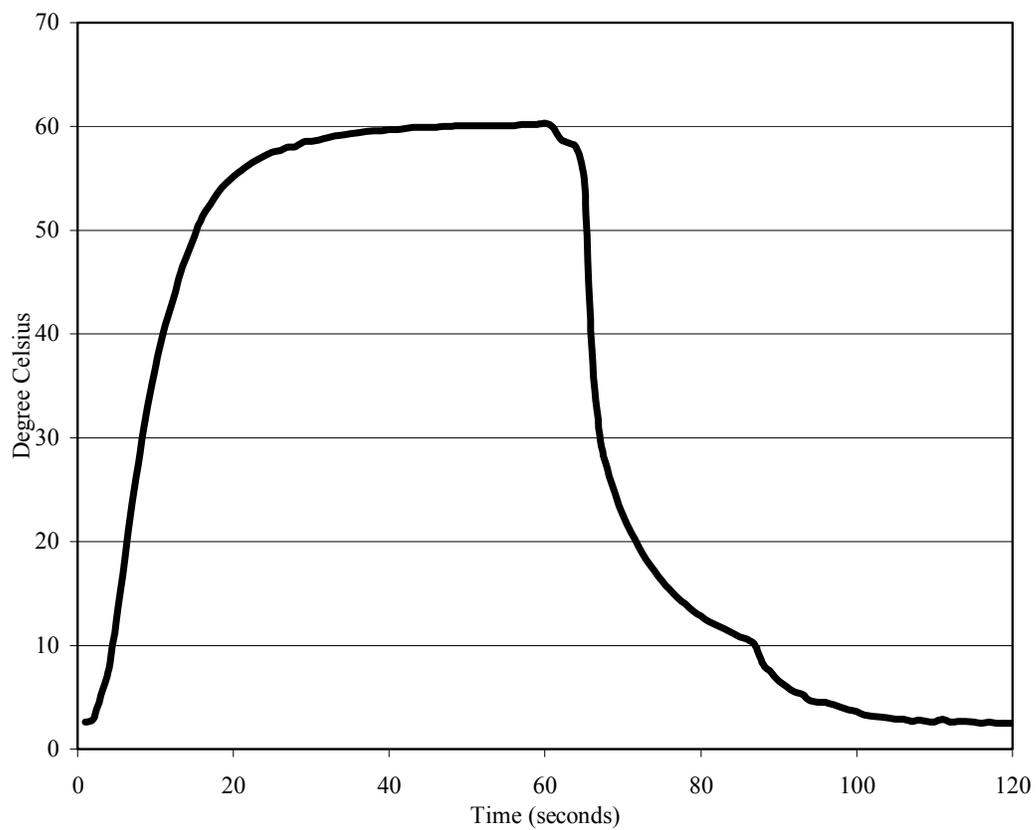


FIGURE 4. Effects of a surface heat pasteurization process in combination with wheat gluten and glycerol films containing nisin, chelators, and a surfactant on surviving populations of *E. coli* O157:H7 recovered from the surface of a low fat turkey bologna (Control – no film; Film – film without treatment components; Film+N – films containing nisin and other treatment components; with linear regression equations).

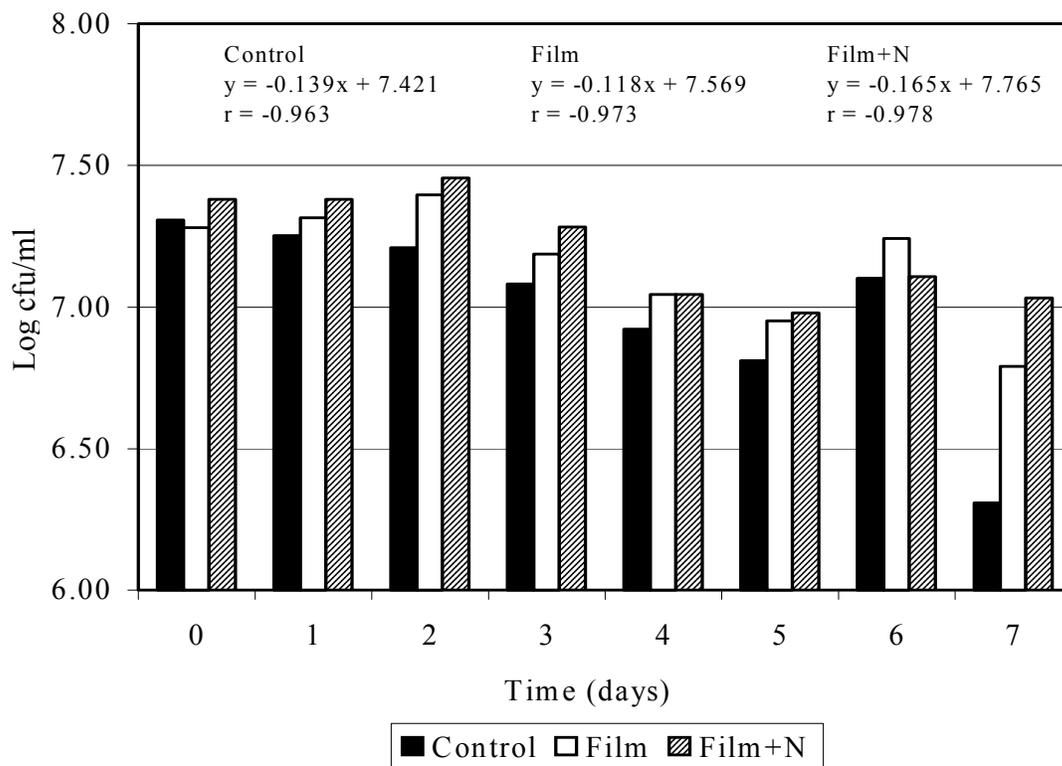


FIGURE 5. Effects of a surface heat pasteurization process in combination with wheat gluten and glycerol films containing nisin, chelators, and a surfactant on surviving populations of *C. jejuni* recovered from the surface of a low fat turkey bologna (Control – no film; Film – film without treatment components; Film+N – films containing nisin and other treatment components; with linear regression equations).

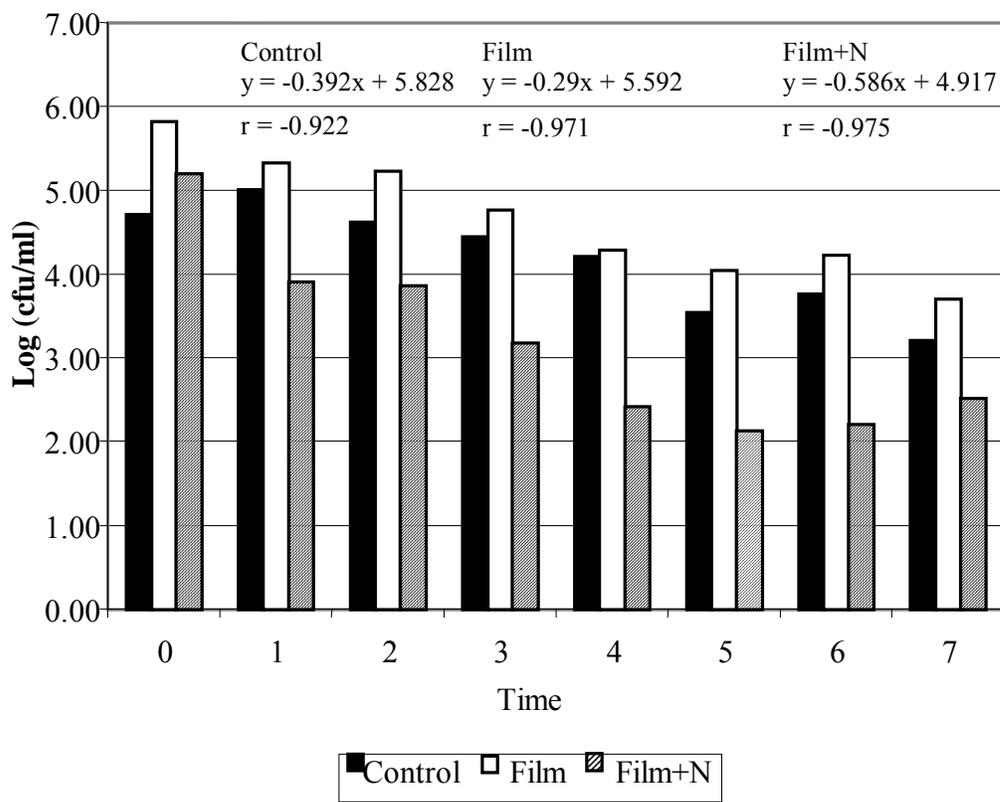


FIGURE 6. Effects of a surface heat pasteurization process in combination with wheat gluten and glycerol films containing nisin, chelators, and a surfactant on surviving populations of *E. coli* O157:H7 recovered from the surface of a low fat turkey bologna stored under refrigeration for 5 weeks (Control – no film; Film – film without treatment components; Film+N – films containing nisin and other treatment components; with linear regression equations).

