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Lactate Dehydrogenase as Indicator of Proper Heat Processing and Death of Escherichia coli 0157:H7 and Salmonella in Ground Beef Patties

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Alicia Orta-Ramirez

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James F. Price
Major professor

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LACTATE DEHYDROGENASE AS INDICATOR OF PROPER HEAT PROCESSING AND DEATH OF *Escherichia coli* O157:H7 AND *Salmonella* IN GROUND BEEF PATTIES

by

Alicia Orta-Ramirez

A THESIS

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ABSTRACT

LACTATE DEHYDROGENASE AS INDICATOR OF PROPER HEAT PROCESING AND DEATH OF *Escherichia coli* O157:H7 AND *Salmonella* IN GROUND BEEF PATTIES

by

Alicia Orta-Ramirez

Three media (3M Petrifilm™ Coliform Plate and *E.coli* Plate Counts, and McConkey-Plate Count Agar, 50: 50) were compared for their ability to enumerate *E.coli* O157:H7. No significant differences were found. For convenience Coliform Plate Counts were chosen for heat inactivation studies. Thermal inactivation of *E.coli* O157:H7 in ground beef at 53°, 58°, 63° and 68°C was compared to those of *S.senftenberg* and lactate dehydrogenase (LDH). LDH was the most heat resistant at all temperatures suggesting that it could be used as indicator of proper heat processing in ground beef. Ground beef patties containing 13.6% fat were cooked to internal temperatures of 62.8°, 65.6°, 68.3° and 71.1°C. Ground beef patties containing 10.7% or 19.0% fat were cooked to an internal temperature of 68.3°C. A sandwich ELISA using both anti-LDH monoclonal and polyclonal antibodies was performed on extracts of patties. Significant differences in LDH concentration were found between patties cooked to 62.8°C and both uncooked patties and patties cooked to 65.6°C, but no significant differences were found among patties cooked to internal temperatures of 65.6°, 68.3° and 71.1°C. No significant differences were found among patties containing different fat levels and cooked to 68.3°C. High variation among patties cooked under same conditions interfered with detection of LDH. This problem should be addressed before attempting further studies.

This is dedicated to my parents, Carlos Orta and Milagros Ramirez, and my sisters, Nuria, Gemma and Teresa, without whom I would not be the person I am. Also to my parents in-law, Enric Garcia and Rosa Mª. Sirera, without whom I would not be here, and to Josep for his love and encouragement.

Finally, I would like to dedicate this work to the people in my life that make me smile.

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INTRODUCTION

Recent outbreaks of *Escherichia coli* O157:H7 (*E.coli* O157:H7) associated mainly with consumption of undercooked ground beef patties, have led to a severe review of the methodology used to ensure safety of cooked meat products. In January of 1994, the Food and Drug Administration (FDA) published new thermal processing recommendations for meat products in a revised version of the Food Code. Comminuted meats should now be processed to an internal temperature of either 63°C (145°F) for 3 min, 66°C (150°F) for 1 min or 68°C (155°F) for 15 sec. According to the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) regulations, effective as of September 1, 1993, official establishments that manufacture fully-cooked patties will have to use any of seven time/temperature schedules with holding time. Official establishments that manufacture partially-cooked patties will have to raise the internal temperature to a minimum of 60°C (140°F) and then cool to a maximum temperature of 4°C (40°F) within 2 h. (USDA-FSIS, 1993).

Current methods employed by USDA-FSIS to verify proper heat processing of meat products are considered subjective and do not provide an adequate margin of safety. There is an urgent need for rapid and accurate assays that ensure safety of cooked meat products.

Determination of the endpoint temperature to which a product has been cooked is a common way to verify compliance to regulations. The use of

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enzyme markers to assess the endpoint cooking temperature in meat products has already been suggested. Once a potential marker has been identified, it can be detected by different methods including residual enzymatic activity, solubility and immunoassays. Among them, enzyme-linked immunosorbent assays (ELISA) are gaining importance due to their specificity, sensitivity and ease of application.

The purpose of this research was to develop an ELISA to verify proper thermal processing of ground beef patties. From previous work (Abouzied et al., 1993; Wang et al., 1994), lactate dehydrogenase (LDH) had already been identified as a potential marker. The first objective was to evaluate and compare three different media for enumeration of *E.coli* O157:H7. The second objective was to compare thermal inactivation of LDH, *E.coli* O157:H7 and *Salmonella senftenberg* in ground beef at four different temperatures. A third objective was to produce monoclonal antibodies against bovine muscle LDH. The final objective was to verify endpoint cooking temperature of ground beef patties by a sandwich ELISA using polyclonal and monoclonal antibodies against LDH.

1. LITERATURE REVIEW

Microbial contamination is considered the most important hazard associated with foodborne illnesses. According to Todd (1989), microbial diseases accounted for 84% of all foodborne disease costs in the United States, with a total estimated cost of $8.4 billion and an average of $670 per case. More recently, Todd (1994) estimated the number of cases resulting from foodborne infections and intoxications to be 5.5-6.2 million costing $5.8-8.6 billion.

Inadequate cooking of meat products and improper storage or holding temperatures seem to be the most common errors that lead to foodborne outbreaks (Bean and Griffin, 1990). During the last decade, severe outbreaks of food poisoning due to *E.coli* O157:H7 had become more frequent. The number of cases of diarrhea caused by this microorganism is estimated to be 10,000-20,000 per year. Estimated costs of medical treatment and lost productivity account for $216-580 million per year (Anonymous, 1994). Because of the high correlation of these outbreaks with consumption of undercooked beef products and because of the severity of the clinical presentations such as hemolitic-uremic syndrome and thrombotic thrombocytopenic purpura, it has become a real concern for consumers, the meat industry and government regulatory agencies. As a consequence, FDA and USDA have reviewed the current regulations concerning cooking temperatures of meat products.

1.1. Meat safety considerations

Title 9 of the Code of Federal regulations outlines the requirements for the processing of meat products needed for destruction of pathogenic

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microorganisms that can cause foodborne disease (USDA-FSIS, 1990). Cooked beef and roast beef are required to be heat processed in any of 16 time/temperature schedules with holding time or to 63°C (145°) with no holding time. Pork products are required to be processed to an internal temperature of 58.3°C (137°F) or subjected to freezing or drying to ensure proper destruction of the parasite *Trichinae*. Uncured and cured/smoked poultry products are required to be processed to internal temperatures of 71.1 °C (160°F) and 68°C (155°F), respectively.

After the 1993 outbreak of *E.coli* O157:H7 associated with consumption of undercooked ground beef patties that involved more than 500 people, including 4 deaths, the government regulatory agencies have reviewed and updated the current thermal processing regulations.

In January 1994, the FDA published a new Food Code (FDA, 1993) establishing new recommendations for cooking meat products. From now on, pork products and conminuted meats shall be cooked to internal temperatures of either 63°C (145°F) for 3 min, 66°C (150°F) for 1 min or 68°C (155°F) for 15 sec. Roast beef and corned beef shall be cooked to any of the following time/temperature protocols: 54°C (130°F)/121 min, 56°C (132°F)/77 min, 57° C (134°F)/47 min, 58°C (136°F)/32 min, 59°C (138°F)/19 min, 60°C (140° F)/12 min, 61°C (142°F)/8 min, 62°C (144°F)/5 min or 63°C (145°F)/3 min. Poultry products shall be cooked to 74°C (165°F) or above for 15 sec. In addition, according to USDA-FSIS regulations, official establishments that manufacture fully-cooked patties shall use one of the following time/temperature protocols: 66.1°C (151°F)/41 sec, 66.7°C (152°F)/32 sec, 67.2°C (153°F)/26 sec, 67.8°C (154°F)/20 sec, 68.3°C (155°F)/16 sec, 68.9°C (156°F)/13 sec, and 69.4°C (157°F) (and up)/10 sec. Also, official establishments that manufacture partially cooked patties are required to heat

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patties to a minimum internal temperature 60°C (140°F) followed by cooling to a maximum internal temperature of 4°C (40°F) within 2 h (USDA-FSIS, 1993).

Although the increase in temperatures requirements can help reduce the incidence of foodborne disease, there is still the problem of confirming adequate cooking once the product has been processed. In two outbreaks of *E.coli* O157:H7 (Michigan and Oregon, 1982) cooler spots on the grill were detected during times of peak use (Griffin and Tauxe, 1991), with subsequent inadequate heating of some of the hamburgers.

1.1.1. Current USDA methodology

The USDA-FSIS currently employs three different assays to verify proper thermal processing of meat products. The residual Acid Phosphatase test (USDA-FSIS, 1986a) is used for canned hams, picnics and luncheon meats. This assay is based on the residual enzyme activity in water-soluble protein extracts of cooked samples. Positive reactions show a blue color that can be read at 610 nm. One problem with this assay is that it has been demonstrated that there is a great loss in acid phosphatase activity during frozen storage (Townsend, 1989). This should be taken into consideration when analyzing samples that have been kept under freezing conditions.

The Coagulation test (USDA-FSIS, 1986b) is used for beef and pork products. The method is based on loss of protein solubility during heat processing of the meat product. Protein extracts of cooked samples are heated until cloudiness appears. The temperature at which the turbidity occurs is considered the internal temperature the product reached during cooking. For products cooked to the 63°-71°C the assay can differ 8°-10°C from the real temperature achieved during processing (Townsend and

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Blankenship, 1989). This test is thus very subjective and only gives a gross estimation of the thermal process applied.

The Bovine Catalase test (USDA-FSIS, 1989) is used to verify roast beef and cooked beef. The assay is based on the production of foam when catalase reacts with oxygen. Bovine catalase is destroyed in the 60.5°- 61.1° C temperature range. With the increase of cooking temperature requirements a negative sample is likely to have been undercooked by at least 2°C. In addition, the visual detection of foam is very subjective. None of these assays are sensitive and reliable. There is urgent need for objective and accurate assays to verify adequate thermal processing.

1.1.2. Alternative methodology

The need to assess proper heat processing of meat products to ensure absence of pathogenic bacteria and viruses has led to increased emphasis in the search for a sensitive method that accurately determines the temperature to which a meat product has been cooked. Several approaches have been suggested which can be categorized in two groups: (1) those using an enzyme system as indicator of internal temperature achieved during thermal processing, and (2) those based on physical techniques.

Different enzymes have been tested for their potential as indicators of endpoint temperature (EPT) of cooking. Davis et al. (1988) reported loss of pyruvate kinase activity in both a model system and a commercial canned cured pork product. The enzyme became inactive between 69.5° and 70°C. The method was based on the loss of fluorescence when NADH is oxidized which could be observed under long-wave ultraviolet light. The assay, however, requires visual determination, thus likely to be subjective. McCormick et al. (1987) used a reverse phase high performance liquid

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chromatography to monitor the effect of heating on porcine extracts. Townsend and Blanckenship (1987) used the APIZYM enzyme system to monitor decrease in enzymatic activity in heated aqueous and saline extracts. The APIZYM system can detect 19 different enzymatic activities in a variety of specimens. As a result, the authors suggested that the enzyme leucine aminopeptidase had potential to act as indicator of EPT. The method, however, was not tested in whole muscle samples. Bogin et al. (1992) assayed the activity of 12 enzymes in turkey breast samples heated to different temperatures and they concluded that of the 12, L-aspartate-2-oxoglutarate aminotransferase, creatine phosphotransferase, isocitric dehydrogenase, aldolase, malate dehydrogenase, and lactate dehydrogenase could be used as indicators for verification of heat treatment. Townsend and Davis (1992) evaluated the potential of glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GTP) as indicators of EPT in ground beef using a commercial test kit. Low values of GTP activity in all samples indicated that the enzyme could not be used as an indicator, whereas because of relatively high activity values for GOT in products cooked to 71.1 °C it was suggested that this enzyme might have potential in meat products that have to be cooked to very high EPT. Kormendy et al. (1992) presented a modified acid phosphatase assay and suggested that it could be successfully used to verify heating in canned hams. Townsend et al. (1993) reported loss in N-acetyl-β-D-glucosaminidase activity in samples of beef, pork and poultry heated from 40° to 70°C. Hsu (1993) screened enzymatic activity of 26 enzymes from extracts of turkey muscle for their suitability as indicators of proper heat processing. Of the 26, the author reported LDH and malate dehydrogenase to have potential to be used as markers in poultry products. Townsend et al. (1994) studied creatine phosphokinase activity as a possible

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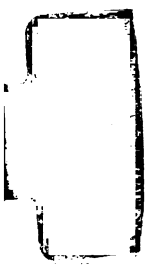
indicator of EPT using a commercial test kit. Although enzymatic activity in meat and poultry products was reduced with increasing processing temperatures, the test was very product dependent, and the authors suggested that it should not be used as a regulatory assay. Wang, Abouzied and Smith (1994) screened different enzymes to be used as indicators of proper heat processing in ground beef patties, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and enzymatic assays. They concluded that triosephosphate isomerase, LDH and phosphoglycerate mutase showed potential to be used as EPT indicators.

LDH is probably the most thoroughly studied enzyme for its potential as indicator of adequate thermal processing. Collins et al. (1991a) measured LDH activity in samples of beef muscle cooked to different temperatures. They observed a sharp decline when samples were heated to 63°C and only marginal activity could be detected at 66°C. The same authors evaluated LDH in cured and uncured pork products (Collins et al., 1991b). For uncured products, activity decreased markedly at 63°C and was almost undetectable at 68°C. For cured products no activity was detected at either 63° or 68°C. Stalder et al. (1991) studied the effect of pH, salt, phosphate, cooking temperature, muscle variation, carcass sex and maturity on LDH activity from heated beef extracts. The authors concluded that LDH showed potential to be used as an EPT indicator. In a different approach, Wang et al. (1992) developed an indirect competitive ELISA to verify proper cooking of uncured turkey breast rolls based on detection of LDH using polyclonal antibodies. The ELISA accurately distinguished EPT within ±1.1-1.2°C in the temperature range of 68.3° to 72.1°C. In a related study, Abouzied et al. (1993) developed monoclonal antibodies to be used in a sandwich ELISA. Minimum detection limits for turkey and chicken LDH were 1 ng/mL. Effects



of formulation, storage and processing of poultry products on LDH were also studied (Wang, 1993). Salt concentration, cooking protocol and type of casing did not markedly influence LDH content.

Another approach to monitor EPT reached during thermal processing of meat products is the use of physical techniques. These include: electrophoretic separation, differential scanning calorimetry (DSC), near infrared spectroscopy (NIS), loss of protein solubility and determination of color.

Caldironi and Bazan (1980) used SDS-PAGE to monitor disappearance of low-salt soluble proteins when beef muscle samples were heated at different temperatures. They observed a gradual loss in some bands after reaching 60°C until their complete disappearance above 80°C. Steele and Lambe (1982) used SDS gradient gel electrophoresis to separate polypeptides formed during heating of different muscle extracts. The intensity of the bands decreased with increasing heating temperatures.

Isaksson et al. (1989) observed changes in spectra of samples of beef with increasing temperature when analyzed by DSC. In another study, samples of beef muscle heated at five different temperatures were studied using DSC (Ellekjaer, 1992). The method very accurately determined the temperature to which the samples have been cooked (±0.6°C). Ellekjaer and Isaksson (1992) verified thermal processing of beef samples that had been cooked to nine different temperatures using near infrared reflectance and transmittance. Both methods could detect EPT within 2.0°-2.1°C.

Davis et al. (1985) measured the amount of water-extractable proteins in pork and beef muscles heated following different time/temperature schedules using the biuret method. They observed that loss of solubility was time/temperature dependent but concluded that more information was needed

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to determine a extractable biuret-positive ratio to be used in a mathematical model. In a related study, Davis et al. (1987) monitored loss of protein solubility in canned cured pork by isoelectric focusing on polyacrylamide gels. Again the authors remarked about the difficulty of establishing a method to verify EPT based on a measure of extractable protein.

Determination of color of cooked meat products has also been suggested by some authors. Ang and Huang (1994) studied color changes in chicken leg patties cooked to different EPT. Although some differences could be seen among patties, both packaging method and storage time influenced markedly the results of color values. Hague et al. (1994) evaluated color of ground beef patties cooked from 55° to 77°C. Internal color lost redness with increasing temperature. Some patties, however, showed premature browning. In addition, visual evaluation proved to be not very accurate.

The main disadvantages in some of these methods are that they require expensive equipment and well trained personnel, or are very time consuming.

1.2. *Escherichia coli* O157:H7

Escherichia coli (*E.coli*) is a microorganism commonly found in the intestinal tract of humans and most mammals. A few strains, however, are pathogenic and can cause gastrointestinal syndromes. These pathogenic strains have been categorized according to their virulence, clinical presentation, epidemiology and O:H serogroups, and include: enteropathogenic *E.coli*, enterotoxigenic *E.coli*, enteroinvasive *E.coli*, and enterohemorrhagic *E.coli*. Among them enterohemorrhagic *E.coli*, which includes *E.coli* O157:H7 and *E.coli* O26:H11 (Padhye and Doyle, 1992) has

become very important due to its role in several foodborne disease outbreaks during the last decade.

1.2.1. Characteristics of the microorganism

E.coli O157:H7 shows most typical biochemical features of *E.coli* except sorbitol fermentation and β-glucuronidase activity. *E.coli* isolates ferment sorbitol within 24 h, whereas *E.coli* O157:H7 failed to ferment sorbitol within 7 days (Wells et al., 1983). In addition, this microorganism has been shown to be negative in the 4-methylumbelliferyl β-D-glucuronide (MUG) assay. This test, used to detect *E.coli*, is based on the hydrolyzation of MUG which results in a fluorogenic compound (Doyle and Schoeni, 1984).

E.coli O157:H7 grows well at 30-42°C with optimum growth at 37°C. This microorganism grows poorly between 42° and 45°C and does not grow at all at 4°, 10° and 45.5°C (Doyle and Schoeni, 1984). Common procedures for fecal coliform enumeration use incubation temperatures of 44-45.5°C. Therefore, being unable to grow at these temperatures, *E.coli* O157:H7 cannot be detected using standard coliform procedures (Doyle and Schoeni, 1984; Raghubeer and Matches, 1990).

Studies of thermal inactivation of *E.coli* O157:H7 in ground beef containing 17-20% fat and heated to 54.4°, 57.2°, 58.9°, 60°, 62.2°, 62.8° and 64.3°C resulted in D values of 39.8, 4.5, 1.2, 0.8, 0.4 and 0.2 sec, respectively (Doyle and Schoeni, 1984). In a similar study using ground beef containing 2% fat, Line et al. (1991) reported D values of 78.2-80.1, 4.1-4.0 and 0.30-0.22 min when heated to 51.7°, 57.2° and 62.8°C, respectively, using two different recovery methods.

When ground beef patties inoculated with *E.coli* O157:H7 were frozen at -80°C and held at -20°C, there was little change in population after 9

months of storage, suggesting that this microorganism can survive well in freezing conditions (Doyle and Schoeni, 1984). In studies of pH tolerance, Abdul-Raouf et al. (1993) found that *E.coli* O157:H7 populations did not change in beef slurries at pH 4.70 after 24 h of storage at 5°C. In addition, they observed growth in slurries acidified to 4.70 with either citric or lactic acid after 24 h at 30°C. Weagant et al. (1994) reported more extended survival times for *E.coli* O157:H7 in mayonnaise (pH 3.65) held at 5° or 7° than at 25°C. Thayer and Boyd (1993) studied the effect of irradiation on *E.coli* O157:H7 in ground beef and chicken meat. The microorganism was sensitive to gamma radiation at the 1.5-3.0 kGy dose range.

1.2.2. Clinical presentations

Three major syndromes have been associated with *E.coli* O157:H7: hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TPP).

Hemorrhagic colitis is characterized by severe abdominal pain followed by watery diarrhea which progresses to a bloody discharge. Vomiting can also occur but none or slight fever is observed (Riley, 1987; Cohen and Giannella, 1991; Padhye and Doyle, 1992). Clinical examination reveals intestinal surface ulceration with marked edema in the lamina propia, variable neutrophilic infiltration, and mucosal thickening in some cases (Neill et al., 1987). The incubation period is approximately 3-4 days with an average duration of 7 days. The microorganism can be detected in patients feces within a week of the onset of symptoms, after which isolation becomes more difficult or not possible. It is believed that some risk factors exist, such as age (children and elderly are most susceptible), use of antibiotics and previous gastrectomy (Griffin and Tauxe, 1991). The treatment of HC is

based on alleviating dehydration and replenishing electrolyte abnormalities and blood loss. The use of antibiotics is controversial. According to Tarr et al. (1988), antibiotic treatment can increase the risk of HUS by two mechanisms: (a) by depleting competitive gut microflora and (b) by lysis of *E.coli* O157:H7 bacterial cell with subsequent increase of toxin liberation into the intestinal lumen. However, as mentioned by Griffin and Tauxe (1991), in other studies no correlation could be found between the administration of antibiotics and progression to HUS.

Hemolytic uremic syndrome most commonly affects children between 1 and 4 years old. Initial symptoms are those of enterocolitis followed by pallor, vomiting, macroscopic hematuria or oliguria. Clinical examination reveals microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Additionally, hypertension hepatosplecnomegaly and purpura, and leukocytosis may be seen. Complications include central nervous system symptoms such as seizures, and gastrointestinal and cardiac symptoms. The treatment of HUS is based on maintenance of a normal fluid and electrolyte balance, and careful monitoring (Robson and Leung, 1990). Usually, patients require dialysis and blood transfusions, even renal transplantation. Permanent renal or neurologic damage can result. Death may occur in some cases (Cohen and Giannella, 1991; Padhye and Doyle, 1992). As in HC, administration of antibiotics seems to be contraindicated.

Thrombotic thrombocytopenic purpura is similar to HUS because of the vascular histopathology involved, but it occurs more frequently in adults. Clinical features include hemolytic anemia, severe thrombocytopenia, neurologic symptoms such as seizures, drowsiness, confusion and fluctuating levels of consciousness, and fever (Kovacs et al., 1990). Blood clots can be developed in the brain resulting in death (Padhye and Doyle, 1992).

Reported risk factors for developing both HUS and TPP include age, female sex, mental retardation, fever, and use of antibiotics (Griffin and Tauxe, 1991).

Less frequent clinical presentations are hemorrhagic cystitis and balanitis (Gransden et al., 1985), convulsions and anemia (Padhye and Doyle, 1992).

1.2.3. Epidemiology

E.coli O157:H7 was first isolated in California in 1975 in a patient with severe bloody diarrhea, but it was not until 1982, after two major outbreaks in Michigan and Oregon, that it was recognized as a foodborne pathogen microorganism (Padhye and Doyle, 1992). The people implicated in both outbreaks had consumed ground beef hamburgers from the same fast-food restaurant chain. Between 1984 and 1990, ten more outbreaks were reported in Nebraska, North Carolina, Washington, Utah, Wisconsisn, Minnesota, Missouri, North Dakota and Montana (Griffin and Tauxe, 1991). In six out of the ten outbreaks, beef products were identified as the vehicle of contamination. Data from another source (Anonymous, 1994) points out an estimated number of 2-3 outbreaks per year during the period 1982-1992. Outbreaks have also been reported in Canada, United Kingdom, Mexico, Argentina, China and Belgium. More recently, between November 15, 1992 and February 28, 1993, four states (Washington, California, Nevada and Idaho) were involved in the largest outbreak of *E.coli* O157:H7 in the US, which affected a total of 527 people and four children died (Dorn, 1993). Evidence was found that implicated undercooked contaminated hamburgers from a fast-food restaurant.

The mode of transmission is mostly by ingestion of food contaminated with *E.coli* O157:H7. As mentioned before, the most common vehicle implicated in all outbreaks was ground beef patties that had been underprocessed. Borczyk et al. (1987) isolated *E.coli* O157:H7 from fecal samples of healthy calves and cows after an outbreak due to consumption of raw milk, providing evidence that dairy cattle may be a primary reservoir of this microorganism. During slaughter procedures or milking, meat and milk can become contaminated, thus acting as vehicles of *E.coli* O157:H7 poisoning. The microorganism has also been isolated from retail samples of beef, pork, lamb and poultry (Doyle and Schoeni. 1987), suggesting that it is not a rare contaminant of fresh meat and poultry. Other foods implicated in other outbreaks were water, salad dressing, potatoes and apple cider. Although *E.coli* O157:H7 seems to be mostly a foodborne pathogenic microorganism, person-to-person transmission, possibly through a direct fecal-oral route, has also been described in some cases (Ratnam et al., 1986; Griffin and Tauxe, 1991).

The mechanism of pathogenesis is not yet fully understood. Virulence seems to be due to adherence and attachment to intestinal mucosal cells and the production of toxins. Attachment to the mucosa allows the microorganism to resist peristaltic movement and brings toxins into closer contact with mucosal cells, causing damage to the intestinal wall (Cohen and Giannella, 1991). *E.coli* O157:H7 produces at least one or two toxins, called Shiga-like toxins because of their resemblance in structure and activity to the Shiga toxin produced by *Shigella dysenteriae* type 1 (Griffin and Tauxe, 1991). Shiga-like toxin I can be neutralized with antiserum against Shiga toxin, whereas Shiga-like toxin II cannot (Doyle, 1991). The mechanism of action of Shiga toxin and both Shiga-like toxins is almost identical. They all

interfere in protein synthesis in mammalian cells by cleaving an N-glycoside bond at a specific adenine residue of ribosomal RNA. The role of these toxins in the pathogenesis of the disease is not that clear. There seems to be both cytopathic action on the intestinal mucosa and systemic effects affecting extraintestinal vasculature (Cohen and Giannella, 1991). Robson and Leung (1990) reported that these toxins caused the endothelial damage observed in renal arterioles of children with HUS. Both, Shiga-like toxin I and II, are cytotoxic to Vero cells. Hence, another way to designate them is Verotoxin 1 and 2, respectively.

1.2.4. Control and prevention

After more than a decade since the first outbreak directly attributed to *E.coli* O157:H7, there is still insufficient epidemiological and ecological information to outline control strategies to pursue for control and prevention against this pathogenic microorganism. It is to be pointed out, however, the position of three different entities: the National Live Stock and Meat Board, the American Gastroenterological Association Foundation and the USDA.

In 1994, the National Live Stock and Meat Board published the Final Report of the Blue Ribbon Task Force with the aim of supplying directions to improve meat safety based on information provided by experts from the government, academia and industry. The general approach is based on a thorough analysis of nine critical points during the beef production process or, as they called it, "from farm to table". The nine critical areas include: (1) pre-harvest, (2) beef carcass conversion process, (3) beef carcass break-up and trim generation, (4) ground beef processing, (5) food service, (6) retail, (7) public health/consumer education, (8) pathogen reduction/intervention strategies, and (9) regulatory opportunities/challenges. The Task Force

suggested implementation and evaluation of Hazard Analysis and Critical Control Points (HACCP) system in each of the nine steps of the production chain, promotion of research to identify reservoirs of *E.coli* O157:H7 and development of pathogen reduction strategies, approval of antimicrobial rinses for beef carcasses and beef irradiation, and design of consumer education programs on food safety.

In July 11-13, 1994, a panel of experts convened by the American Gastroenterological Association Foundation issued a consensus statement providing recommendations for government, industry and medical professionals for the control and prevention of *E.coli* O157:H7. The panel recommended the implementation of HACCP by food service facilities and retail operations, increase reporting of outbreaks and funding of epidemiological studies. They also recommended the inclusion of *E.coli* O157:H7 screening and detection in stools, besides the microorganisms routinely examined; the implementation of education programs in farms, slaughter and production plants, food service and retail operations, nursing homes and day care providers; education of the medical community on diagnosis and treatment of the disease; and promotion of research on *E.coli* O157:H7 pathogenesis, reservoirs, detection, control and prevention. The panel agreed that, at present, the complete elimination of *E.coli* O157:H7 would only be possible by thorough cooking or irradiation (Anonymous, 1994).

According to an interview with a USDA spokesperson published in the Food Chemical News, July 18, 1994, USDA was about to implement the HACCP systems in all meat and poultry plants. In addition, the agency is following three different approaches: the development of a rapid test for *E.coli* O157:H7 to be used in the plants, the use of a microbial kit so

inspectors can check sanitation of the equipment and facilities, and the increase of research studies of the meat and poultry production chain.

1.2.5. Detection of E.coli O157:H7 in foods

As mentioned before, standard procedures for detection of fecal coliforms are not likely to detect *E.coli* O157:H7 since the test uses incubation temperatures in the range of 44°-45°C. It has been demonstrated that this microorganism is not able to grow at these temperatures. For this reason and because the number of outbreaks due to *E.coli* O157:H7 poisoning has markedly increased during the last decade, researchers have been focusing in the development of methods for detection and isolation of this microorganism in foods. In 1992, Padhye and Doyle published a complete review on the suggested procedures for detection and isolation of *E.coli* O157:H7.

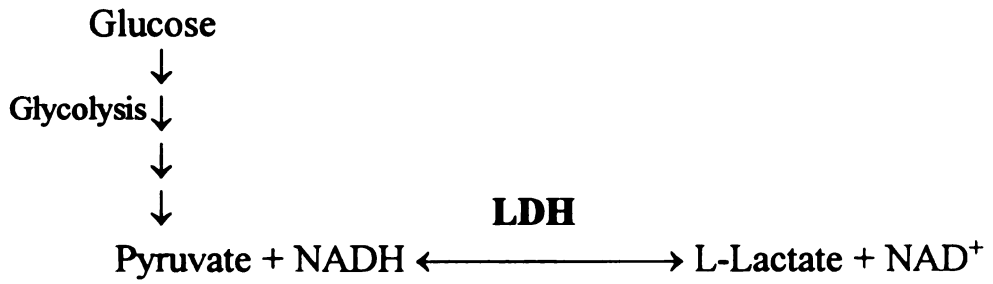
Published methods range from plating on differential media such as MacConkey-sorbitol agar, to the use of sophisticated oligonucleotide probes and colony blot hybridization techniques. With the advent of immunoassays, polyclonal and monoclonal antibodies have been produced against the O157 antigen and two proteins associated with a plasmid. Other procedures include latex agglutination, a specific coliphage, gene and DNA probes and a polymerase chain reaction.

The major flaws in these techniques are the need for incubation period and/or enrichment procedures which are very time consuming, the required use of sophisticated and expensive equipment, and high possibility of cross-reactions with other Enterobacteria and Shiga-like toxin producing *E.coli*.

Another approach could be indirect "detection" of absence of *E.coli* O157:H7 by a marker or indicator which could be somehow correlated with the presence or absence of the microorganism.

1.3. Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is the enzyme involved in the final step of anaerobic glycolysis and reversibly catalyzes the conversion of pyruvate to lactate in the presence of NADH and NAD^+, respectively, according to the following reaction:



The enzyme consist of a group of five tetrameric isoforms which result from the combination of two different polypeptide subunits, H and M, as follows: H_4, H_3M, H_2M_2, HM_3, and M_4 (Holbrook, 1975). The subunits molecular mass is about 35 kD. All five isozymes can exist in most tissues. The H-type subunit, however, predominates in aerobic tissues such as heart muscle, whereas the M-type predominates in skeletal muscle and liver. The H_4 isoform has a low affinity for pyruvate and is inhibited by high levels of this compound, while the M_4 isoform has a much higher affinity and is not inhibited by pyruvate. The other isoforms show intermediate properties according to the proportion of one subunit or the other. Therefore, it has been

suggested that H-type LDH catalyzes the transformation of lactate to pyruvate while the M-type catalyzes the reverse reaction (Voet and Voet, 1990).

LDH has been extensively reviewed for its potential as an indicator of endpoint temperatures in beef (Collins et al., 1991a; Stalder et al., 1991; Wang et al., 1994), pork (McCormick et al., 1988; Collins et al., 1991b) and turkey (Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993; Desrocher, 1994). From previous studies in the Department of Food Science and Human Nutrition at Michigan State University, LDH has been identified as a possible indicator of proper heat processing of ground beef patties (Wang et al., 1994). However, a comparison between thermal inactivation rates of LDH and those of pathogenic microorganisms such as *E.coli* O157:H7 and *Salmonella senftenberg* had never been done before.

1.4. Immunoassays in Food Science

In recent years, immunoassays have been replacing standard methods of food analysis due to their sensitivity, specificity and ease of application. Immunoassays are now being developed to detect adulterants, microorganisms and their toxins, pesticides, and other contaminants in foods (Pestka, 1988; Martin et al., 1988; Samarajeewa et al., 1991). One great advantage of immunoassays is that they can be optimized into a kit to reduce time and instrumentation, so they can be used in the field by non-trained personnel and can be applied to a large number of samples. The two most common immunoassays in the food area are ELISA and radioimmunoassay (RIA). ELISA, however, is more popular because of ease and safety of application.

An ELISA is based on the interaction between an antigen and an antibody. Antibodies (ABs) are proteins secreted by specialized B-lymphocytes, called plasma cells, during an immune response, that bind

specifically to a foreign molecule or antigen (AG). ELISAs can be categorized in three groups: the AB capture assay, the AG capture assay, and the two-AB sandwich assay. In an AB capture assay the AG is immobilized on a solid support and quantitation is done by measuring the amount of labeled AB that binds immobilized AG. In an AG capture assay the same principle applies except that the AB is immobilized and free AG is added to bind the AB. In a sandwich assay, one AB is immobilized on the solid support and free AG is added. Quantitation is done by measuring the amount of a second labeled AB that binds the bound AG (Harlow and Lane, 1988). When choosing an ELISA two factors should be considered: types of ABs that are available, and purity of AG.

1.4.1. Types of Antibodies

As mentioned before, ABs or immunoglobulins (Ig) are proteins synthesized in response to the presence of a foreign substance. Each AB consists of at least four subunits, two identical heavy chains and two identical light chains linked to form a Y shaped molecule. ABs can be categorized in five groups, IgG, IgM, IgA, IgE and IgD, based on their biological activity and type of heavy chain (Burke, 1993).

Properties of an AB are affinity, avidity, titer and specifity. Affinity refers to the precision of fit between AB and the antigenic determinant. The affinity constant for an AB can range from 10^7 to 10^9 M^{-1}, reaching values of 10^{12} M^{-1} in some cases. Since an AB has more than one binding site, avidity refers to the likelihood of a second antigenic determinant binding the AB after the binding of a first site. This term reflects the strengh and stability of the AG-AB complex. The titer is an arbitrary value defined as the dilution showing an optical density that is at least twice the value of the non-immune

serum at the same dilution. Finally, the specificity is the AB ability to recognize more than one molecule.

According to the production method two types of ABs can be made: polyclonal and monoclonal.

1) Polyclonal ABs (PABs)

PABs are produced when different lymphocyte clones respond to the same AG. The resulting immune serum will show a broad range of ABs directed against different antigenic determinants. In short, production of PABs requires immunization of a suitable animal (usually rabbit) with the desired AG, periodical reinjections to induce immune response and collection of immune serum.

Advantages of using PABs are: versatility, stability in frozen or lyophilized conditions, and lower production cost than monoclonals. They are quicker to obtain and can usually be used at higher dilutions (Peters and Baumgarten, 1992). Disadvantages include limited supply, and the fact that extremely pure AG is required to elicit high affinity PABs.

2) Monoclonal ABs (MABs)

Each AB-producing cell has the ability to produce a specific AB against one particular site or epitope of an AG. ABs resulting from a single cell or clone are homogeneous and called MABs. The method to produce MABs involves immunization of a suitable animal (usually mouse) and periodic reinjections to elicit an immune response. Due to the limited life span of the AB-producing cells, it is necessary to find a way to "immortalize" such cells. According to Galfre and Milstein (1981), if B-lymphocytes are induced to fuse with a tumorous lymphoid cell (myeloma), the resulting

hybridoma will grow indefinitely in culture while secreting ABs. Hybridomas can then be separated and cloned and different cell lines can be obtained, each one producing a different MAB against a specific epitope of the selected AG. The MABs can be recovered and purified from the tissue culture supernatant. Another way to produce MABs is by inducing tumor production in the intraperitoneal cavity. MABs can be recovered from the ascites fluid in higher concentration than culture supernatant.

The advantages of using MABs over PABs are their specificity and selectivity for individual antigenic determinants, and unlimited supply. They do not require highly purified AG to elicit a response and can also be used to study functional domains in a molecule. The main disadvantages are: longer time and higher cost of production, higher physicochemical variability and biological activity than PABs and possibility of cross-reactions (Peters and Baumgarten, 1992). In addition, mutations can reduce AB production and cell lines can be lost due to fungal contamination.

1.4.2. Antigens

For an AG to elicit an immune response it has to have a molecular weight of at least 5000 Daltons (Burke, 1993). In this case, the small AG is called a hapten and it has to be conjugated to a large molecule such as bovine serum albumin, to induce AB production.

The antigenic determinant of a native protein must be at the surface of the molecule to be able to be bound by the AB. Studies of antigenicity of native proteins suggest that many antigenic states depend on the secondary, tertiary or even quaternary structure of the molecule, since most of the time they are formed by discontinuous fragments of the polipeptide chain and brought together by folding (Burke, 1993). The same studies show that

native proteins present only a few potential antigenic determinants, but short peptide sequences derived from the native protein can be used to induce ABs against that protein.

2. COMPARISON OF THREE MEDIA FOR ENUMERATION OF *Escherichia coli* O157:H7

2.1. ABSTRACT

Three different media (3M Petrifilm™ Coliform Plate Counts, 3M Petrifilm™ *E.coli* Plate Counts, and McConkey-Plate Count Agar, 50:50) were compared for their ability to enumerate *E.coli* O157:H7. Both, regular and heat-shocked cultures of six strains of *E.coli* O157:H7 in Tryptic Soy Broth were used in this study. No significant differences in mean log CFU/mL were found among the three media for both regular and heat-shocked cultures. These findings indicated that the use of the more convenient dry plating media could be used instead of conventional pre-poured agar plates in thermal inactivation studies.

2.2. INTRODUCTION

Use of Petrifilm™ Aerobic, Coliform and *E.coli* Plate Counts are well recognized and convenient alternatives to standard pour plating methods for enumerating specific bacterial cells. In a project to evaluate the heat resistance of *E.coli* O157:H7, we proposed to use Petrifilm™ Coliform Plate Counts to enumerate cells. Since we were using pure cultures of *E.coli* O157:H7 to inoculate aseptically collected beef, the Coliform and *E.coli* Plate Counts should yield the same result in estimating inoculum population and surviving cells.

While it is recognized that some plating media such as Phenol red sorbitol agar containing 1% pyruvate, appear to be superior for the recovery of heat injured *E.coli* O157:H7 cells (Ahmed and Conner, 1993), the main

objective in this study was to compare a conventional fresh agar pour-plating system employing nutrient media known to support growth of *E.coli* O157:H7 strains, with dry Petrifilm™ plates for use in heat resistance studies.

2.3. MATERIALS AND METHODS

2.3.1. Media

3M Petrifilm™ Coliform Count Plates and *E.coli* Count Plates were purchased from 3M (St. Paul, MN). McConkey-Plate Count Agar (MC-PCA), 50:50, was made by resuspending separately McConkey agar powder (Becton Dickinson) and Plate Count agar powder (Difco) in distilled deionized water following manufacturer instructions and mixing both in equal amounts. The mixture was sterilized at 121°C for 15 min and about 12 mL were dispensed on each sterile Petri dishes (Baxter Health Care Corporation, Mcgaw Park, IL). After dispensing 1 mL of each dilution, samples on MC-PCA were covered with a layer of MC-PCA.

2.3.2. Cultures

E.coli O157:H7 (ATCC 43894) and strains 932, 204P, 505B, CA-1 and E0019, provided by Dr. L. Beuchat, University of Georgia, were maintained by daily transfer in Tryptic soy broth (Difco Laboratories, Detroit, MI). The day before each trial one loopful of each strain was transferred to each of two 16x150 mm culture tubes containing 10 mL of Tryptic soy broth. On the day of the trial one of the tubes was submerged in a Polystat circulator bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, Il) connected to a bath programmer (Model 1268-62, Cole-Parmer) and set at 58.4°C. The temperature in the tube was monitored using a thermocouple

inserted in the center of a control culture tube containing 10 mL of uninoculated Tryptic soy broth. After 20 min at 58°C the inoculated tube was removed and immediately placed in an ice bath until plated within 2 h. This time/temperature treatment was chosen to give approximately a 50% reduction in cell viability.

Both regular and heat-shocked cultures were plated in triplicate on each of the three media. Bacterial counts were determined by decimal dilutions in sterile 0.1% buffered peptone water (Benton Dickinson Microbiological Systems, Cockeysville, MD). All plates were incubated at 35°C for 24 hr. Each study was performed in triplicate.

2.3.3. Statistical Analysis

Mean log CFU/mL comparisons were performed using Student-Neuman- Keul's Multiple range test of the MSTAT statistical program (MSTAT, 1994) with the mean square error term at the 1% level of probability.

2.4. RESULTS AND CONCLUSIONS

Counts for regular *E.coli* O157:H7 culture achieved 10^8 CFU/mL. Counts for equivalent heat-shocked culture achieved 10^4 CFU/mL. When comparing means for the three media, no significant differences were found at the 1% level of probability (Table 2.1) for both regular and heat-shocked cultures. Either dry plating system was at least equal to the use of the poured agar overlayed system.

The use of Petrifilm Count Plates has two advantages over the use of MC-PCA: (1) no media preparation is needed, and (2) enumeration is easier

and more accurate. On the other hand, any of the three media can be used for identification of *E.coli* O157:H7. Since Coliform Count Plates are much cheaper than *E.coli* Count Plates the authors suggest that Coliform Count Plates is the most convenient media for recovery of *E.coli* O157:H7 among the three tested.

Table 2.1. Statistical comparison among mean log CFU/mL for both regular and heat-shocked cultures of *E.coli* O157:H7 across media.

Media / Culture	Mean Log CFU/mL
Petrifilm Coliform / Regular	8.90^{a}
Petrifilm *E.coli* / Regular	8.89^{a}
MC-PCA / Regular	8.82^{a}
Petrifilm Coliform / Heat-shocked	4.69^{b}
Petrifilm *E.coli* / Heat-shocked	4.70^{b}
MC-PCA / Heat-shocked	4.62^{b}

Values with different superscript are statistically different ($p < 0.01$)

3. THERMAL INACTIVATION OF *Escherichia coli* O157:H7 AS COMPARED TO THAT OF LACTATE DEHYDROGENASE AND *Salmonella senftenberg*

3.1. ABSTRACT

Thermal inactivation of *Escherichia coli* O157:H7 (*E.coli* O157:H7) in ground beef at 53°, 58°, 63° and 68°C was compared to that of the enzyme lactate dehydrogenase (LDH) and of a more heat resistant microorganism, *Salmonella senftenberg* (*S.senftenberg*). Inoculated and non inoculated ground beef samples were placed in sterile thermal death time tubes and immersed in a circulating bath then held at the above temperatures for predetermined intervals of time. Bacterial counts were determined using Petrifilm™ Coliform Count Plates and LDH was assayed for enzymatic activity. The D values (in minutes) for *E.coli* O157:H7 and *S.senftenberg* were 46.1 and 53 at 53°; 6.44 and 15.17 at 58°; 0.43 and 2.08 at 63°; and 0.12 and 0.22 at 68°C; with z values of 5.60° and 6.24°C, respectively. Inactivation rates or "D values" for LDH were 543.48, 19.61, and 1.40 min at 58°, 63° and 68°C, respectively, with a "z value" of 3.86°C. The D value for LDH at 53°C could not be determined. The higher D values and lower z value for LDH as compared to either microorganism indicated that the enzyme is less sensitive to heat inactivation than cells of either culture. Indeed, *S.senftenberg* proved to be more heat stable than *E.coli* O157:H7.

3.2. INTRODUCTION

Studies comparing thermal inactivation rates of pathogenic microorganisms to enzymatic inactivation rates have been done previously, mostly in dairy products. Alkaline phosphatase has been used as an indicator

of proper pasteurization of milk based on its parallel inactivation to the pathogen *Mycobacterium tuberculosis* (Kay and Graham, 1933). In addition, Eckner (1992) demonstrated the relationship between alkaline phosphatase inactivation and *Listeria* and *Salmonella* destruction in milk. More recently, Patel and Wilbey (1994) compared the thermal inactivation of the enzyme γ-glutamyltranspeptidase to that of *Enterococcus faecium* in different milk-based systems and concluded that this enzyme could be used as an indicator of proper heat treatments more severe than pasteurization for milk products.

Because of recent outbreaks of *E.coli* O157:H7 due to insufficient cooking of ground beef patties and because current US Department of Agriculture (USDA) methodology is considered unreliable, there has been increased interest in finding more accurate methods to assure safety of cooked meat products. Several enzymes have been suggested as indicators of proper heat processing in meat products. Among them, the enzyme lactate dehydrogenase (LDH) has been thoroughly studied as a potential end point temperature indicator in pork (Collins et al., 1991b), turkey (Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993; Desrocher, 1994) and beef (Collins et al., 1991a; Stadler et al., 1991; Wang et al., 1994). However, no relationship between inactivation rates of LDH and those of pathogenic microorganisms have yet been determined.

The objective of this study was to determine the D and z values of *E.coli* O157:H7, *Salmonella senftenberg* and LDH in ground beef at four different temperatures and to compare their respective thermal inactivation rates.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial cultures

Cultures of *Escherichia coli* O157:H7 (ATCC 43894) and *Salmonella senftenberg* (ATCC 43845) were maintained by daily transfer in tryptic soy broth (Difco Laboratories, Detroit, MI). Each inoculum was prepared from a 24 hr culture containing approximately 10^9 microorganisms/mL. On the day of the experiment each culture was sedimented by centrifuging at 4340 x g for 20 min at 4°C (Sorvall Superspeed RC2-B, Ivan Sorvall Inc, Norwalk, CT) and resuspended to its original volume using sterile 0.1 % buffered peptone water (Becton Dickinson Microbiology Systems, Cockeysville, MD). The inoculums were enumerated by serial dilution in sterile 0.1 % buffered peptone water (Becton Dickinson Microbiology Systems) and plating on Petrifilm™ Coliform Count Plates (3M, St. Paul, MN).

3.3.2. Preparation of meat

Three eyes of the round (semitendinosus muscle), vacuum packaged and less than six days old, were purchased from a local store. After trimming fat and connective tissue, each piece was immersed in boiling water for 10 sec to reduce surface contamination. Using aseptic technique, the outer surface of meat was removed by cutting with a series of sterilized knives and discarded. Under a sterilized grinder and in a aseptic environment, the meat was ground twice through a 3.175 mm diameter grinder plate in a Hobart grinder (Model 84181D, Hobart Mfg. Co., Troy, OH) and then divided into three portions of 200 g each. One portion was inoculated with *E.coli* O157:H7 and one with *S.senftenberg* 24 hr cultures to achieve about 10^7 microorganisms/g meat. The cultures were added dropwise to the ground

meat and distributed thoroughly, kneading the mixture by hand, using sterile latex gloves, for 5 min. The third portion was not inoculated and used for the LDH studies.

3.3.3. Thermal inactivation

From each portion of ground meat, one-gram samples were filled in sterile 10 x 75 mm thermal death time (TDT) tubes using modified 60 cc syringes. The samples were placed at the bottom of each tube and the tubes were sealed using a gas/oxygen flame. The number of tubes varied according to the time-temperature schedules (Table 3.1), based on preliminary results.

The tubes (one set of each *E.coli* O157:H7, *S.senftenberg* and LDH at a time) were placed in a wire rack and immersed in a Polystat circulator bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, Ill.) connected to a bath programmer (Model 1268-62, Cole-Parmer). The temperature was set 0.5°C above the target temperature and monitored using a thermocouple inserted in the center of a control TDT tube containing 1 g of ground meat. The thermocouple was connected to a Solomat MPM 200 Modumeter (Solomat Partners LP, Glenbrook Industrial Park, Stamford, CT). Time and temperature were recorded using a Solomat MPM Logger (Solomat Partners LP) connected to the modumeter. For 53° and 58°C trials, zero time was when the internal temperature reached target temperature (less than 5 min). For 63° and 68°C, zero time was when the internal temperature reached 58°C (less than 5 min).

Three tubes were removed at each time interval and immediately placed in an ice bath. Tubes containing inoculated samples were kept at 4°C and plated within 12 hr. Tubes containing uninoculated samples were kept at

Table 3.1. Time and temperature schedules for thermal inactivation of *E.coli* O157:H7, *S.senftenberg* and LDH

T (°C)	*E.coli* O157:H7	*S.senftenberg*	LDH
53	0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h	0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h	0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h
58	0, 2, 4, 8, 10, 15, 20, 30, 35, and 45 min	0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 min	0, 2, 4, 8, 10, 15, 20, 30, 35, and 45 min
63	0, 60, 75, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 210, and 240 sec	0, 60, 120, 150, 180, 240, 270, 300, 360, 420, 480, and 600 sec	0, 60, 75, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 210, and 240 sec
68	0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 sec	0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 sec	0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 sec

-20°C until analyzed within 8 weeks. Each thermal inactivation study was performed in triplicate.

3.3.4. Bacterial counts

E.coli O157:H7 and *S.senftenberg* counts were determined by decimal dilution of the ground beef samples in sterile 0.1 % buffered peptone water. The samples were homogenized using a Stomacher lab blender (Model 400, Tekmar Company, Cincinnatti, OH) for 2 min and plated in duplicate on Petrifilm™ Coliform Count Plates (3M). Samples of uninoculated ground Meat were plated on both Petrifilm™ Aerobic Count Plates and Coliform Count Plates for determination of possible initial contamination. All samples were incubated at 37°C for 24 hr.

3.3.5. Extraction of LDH from ground beef

After breaking the sealed TDT tubes, each sample was transferred to a scintillation vial. To each sample, 2 mL of cold phosphate buffer saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) were added and the sample was mixed for 1 min using a vortex mixer. The mixture was further stirred for 15 min at 4°C and then centrifuged at 4500 x g for 5 min (Micro-centrifuge, Model 59A, Fischer Scientific, Itasca, IL). The supernatants were collected and kept at 4°C until analyzed within 4 hr.

3.3.6. Determination of enzymatic activity

The enzymatic activity of LDH at 25°C was determined using a LDH diagnostic kit (DG 1340-K, Sigma, St Louis, MO). The determination of

LDH activity is based on the reduction of pyruvate to lactate and simultaneous oxidation of NADH to NAD which results in a decrease in the absorbance at 340 nm. One unit (U) of LDH activity is expressed as the amount of enzyme which catalyzes the formation of 1μmol of NAD/L per minute under specified conditions.

3.3.7. Proximate analysis and determination of pH

Moisture and fat composition of the ground beef were determined using AOAC (1990) methods 950.46B and 960.39, respectively. For determination of the pH, 50 g ground beef were homogenized with 50 mL of distilled water in a Waring blender for 30 sec. The pH of the homogenate was measured using a Corning 145 pH meter (Fisher Scientific). Results are the average value of triplicate analysis of the same sample.

3.3.8. D and z values

Plate counts (CFU/mL) and enzymatic activity (U/g) data were converted to logarithms. Plate counts less than 10 were entered as zeros. Linear regression analyses were conducted using LOTUS 1-2-3 for Windows, Version 1.0 (Lotus Development Corporation, Cambridge, MA). In 53° and 58°C experiments all data points were used to calculate regression analyses. Since time zero was considered when internal temperature of the samples reached 58°C for the 63°C and 68°C experiments, values from the first 150 sec and 20 sec, respectively, were excluded from the regression analyses to avoid the lag phase of the curve. D values (in minutes) were calculated according to the Laboratory Manual for Food Canners and Processors (1968) from the regression curves at each temperature. TDT phantom curves were determined by plotting log of D values *vs* temperature. The z values were

determined as the absolute value of the reciprocal of the slope of the TDT curve.

3.4. RESULTS AND DISCUSSION

3.4.1. Raw ground beef

Proximate analysis and pH of the three semitendinosus muscles used in this study are shown in Table 3.2. Percent of moisture ranged from 70.8 to 74.7. Fat percent ranged from 1.7 to 5.8. The pH of the samples ranged from 6.2 to 6.3.

Samples of raw ground beef were plated to determine bacterial load after aseptic meat collection. No *E.coli* or *E.coli*-like microorganisms were detected. One sample showed total aerobic counts of approximately 10^2 CFU/g meat. The other two samples had less than 10 CFU/g meat. Analysis of LDH enzymatic activity in raw samples revealed mean initial values of 886 U/g meat.

3.4.2. D and z values

Thermal inactivation or survivor curves for *E.coli* O157:H7 and *S.senftenberg* at 53°C are shown in Figure 3.1. D_{53} values for *E.coli* O157:H7 and *S.senftenberg* were 46.1 and 53 min, respectively. Thermal inactivation curve for LDH at 53°C is shown in Figure 3.2. There was no perceptible change in enzymatic activity after 6 h. and the D value was not calculated.

Survivor curves for *E.coli* O157:H7 and *S.senftenberg* at 58°C are shown in Figure 3.3. D_{58} values were 6.45 and 15.2 min for *E.coli* O157:H7 and *S.senftenberg*, respectively. LDH thermal inactivation curve at 58°C is shown in Figure 3.4. Inactivation rate or D_{58} value was 544 min. After 45

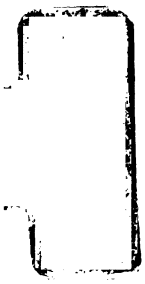


Table 3.2. Results of proximate analysis of raw ground beef samples[a].

Sample #	Moisture (%)	Fat (%)	pH
1	70.8	1.7	6.3
2	73.6	2.5	6.2
3	74.7	5.8	6.3
Mean	73.0±2.01	3.3±2.17	6.3±0.06

[a] Values expressed as mean ± SD.

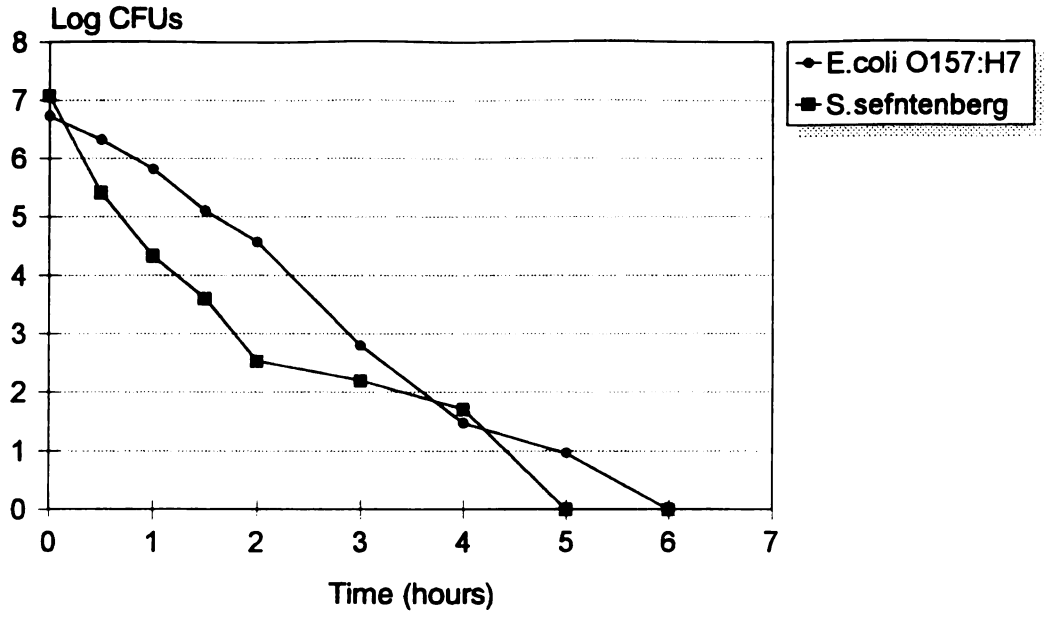


Figure 3.1. Survivor curves of *E.coli* O157:H7 and *S.senftenberg* in ground beef at 53°C.

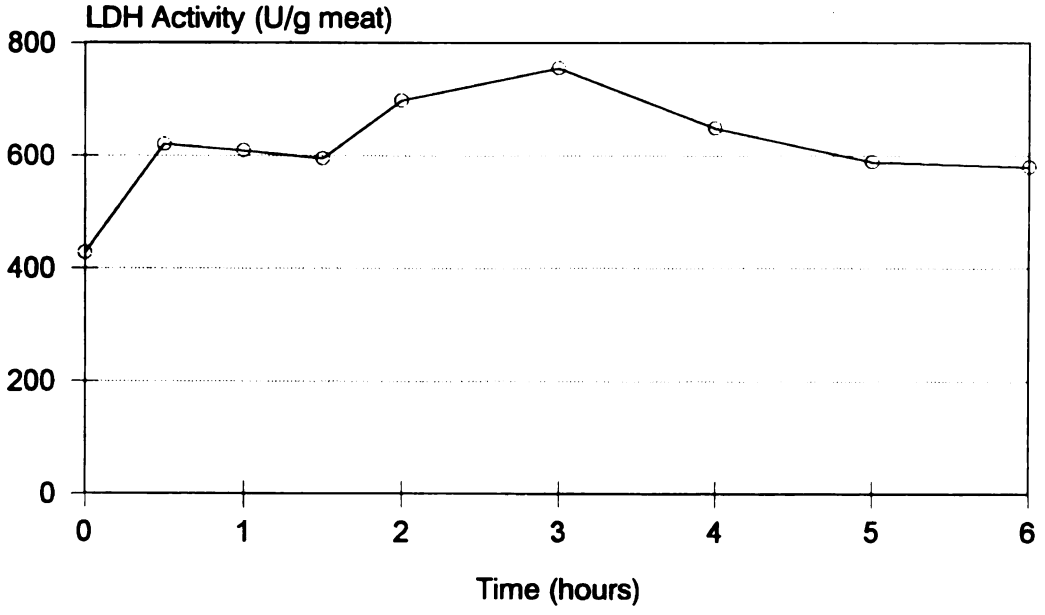


Figure 3.2. Effect of heating on LDH in ground beef at 53°C.

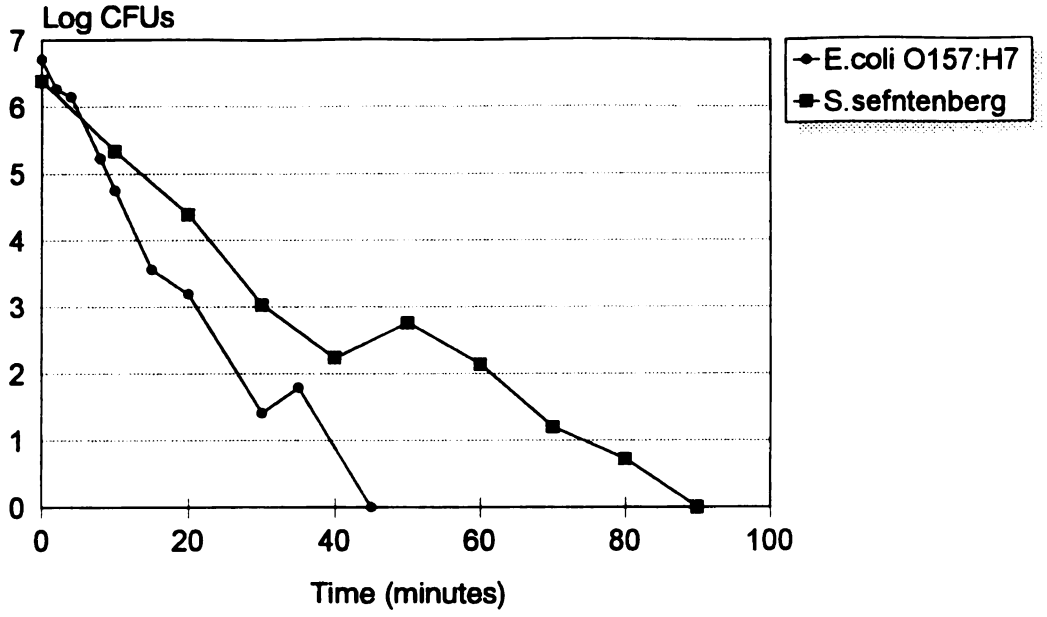


Figure 3.3. Survivor curves of *E.coli* O157:H7 and *S.senftenberg* in ground beef at 58°C.

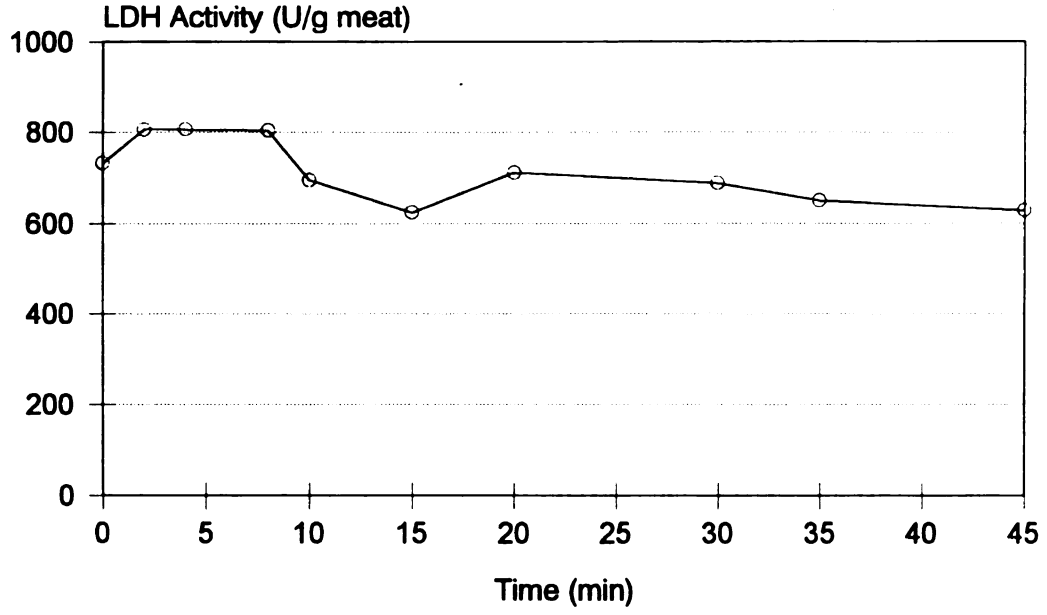


Figure 3.4. Effect of heating on LDH in ground beef at 58°C.

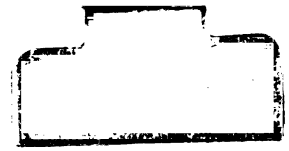
min at 58°C LDH enzymatic activity was reduced by 14% when compared to the raw samples.

Survivor curves for *E.coli* O157:H7 and *S.senftenberg* at 63°C are shown in Figure 3.5. D_{63} values were 0.43 min for *E.coli* O157:H7 and 2.08 min for *S.senftenberg*, respectively. The thermal inactivation curve for LDH at 63°C is shown in Figure 3.6. The D_{63} value was 19.6 min. The enzymatic activity after 4 min at 63°C had been reduced to a 40% of the initial activity.

Survivor curves for *E.coli* O157:H7 and *S.senftenberg* at 68°C are shown in Figure 3.7. At this temperature, D values were 0.12 and 0.22 min for *E.coli* O157:H7 and *S.senftenberg*, respectively. Thermal inactivation curve for LDH at 68°C is shown in Figure 3.8. The D_{68} value was 1.40 min. LDH activity after 90 sec at 68°C was reduced by 89 % of the initial.

Summary of D values and regression analysis at all temperatures for *E.coli* O157:H7, *S.senftenberg* and LDH are listed in Tables 3.3, 3.4 and 3.5, respectively. Summary of z values and regression analysis for *E.coli* 3O157:H7, *S.senftenberg* and LDH are listed in Table 3.6. The lowest z value, 3.86°C, corresponded to LDH. *E.coli* O157:H7 and *S.senftenberg* showed z values of 5.60° and 6.24°C.,respectively. Phamtom thermal death time curves for *E.coli* O157:H7, *S.senftenberg* and LDH were obtained by plotting temperature *vs.* log D values (Figure 3.9).

Doyle and Schoeni (1984) and Line et al. (1991) reported D and z values for *E.coli* O157:H7 in ground beef over a similar range of temperatures as in our study (Table 3.7). Doyle and Schoeni determined D values at 54.4°, 57.2° and 62.8°C of 39.8, 4.5 and 0.4 min, respectively, with a z value of 4.1°C. Line et al. (1991) reported D values at 51.7°, 57.2° and 62.8°C based on two methods of recovery. Their results were 78.2 and 80.1, 4.1 and 4.0, and 0.30 and 0.22 min, with z values of 4.61° and 4.33°C,



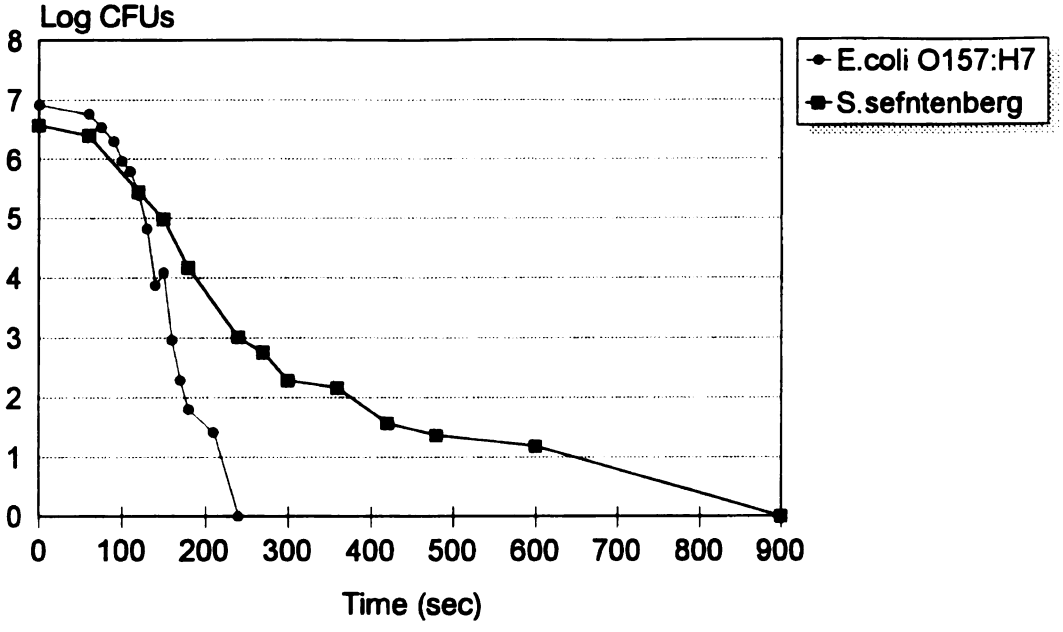


Figure 3.5. Survivor curves of *E.coli* O157:H7 and *S.senftenberg* in ground beef at 63°C.

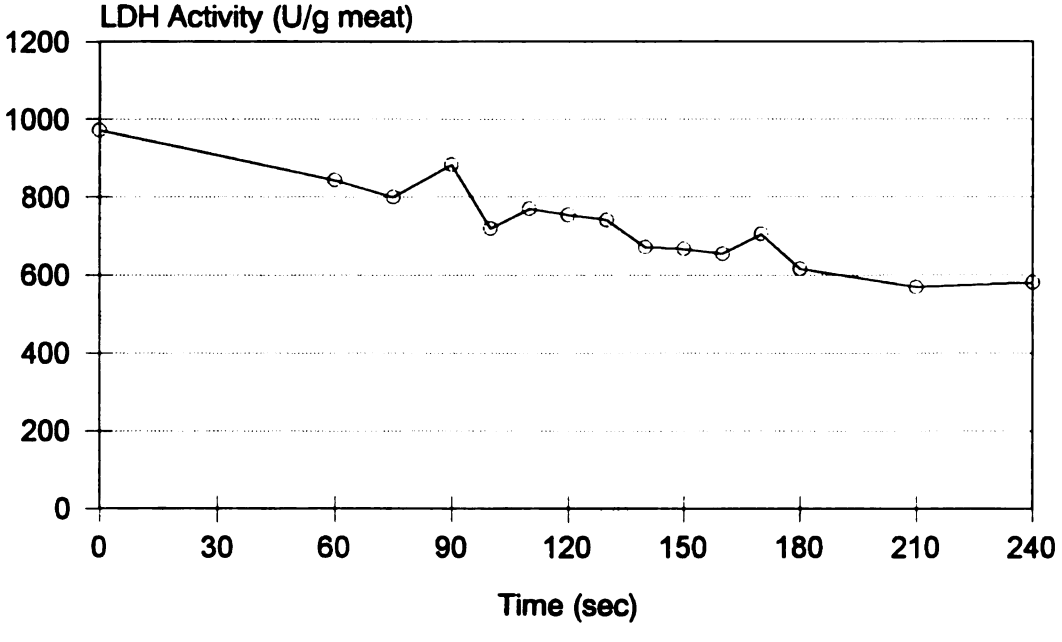


Figure 3.6. Thermal inactivation of LDH in ground beef at 63°C.

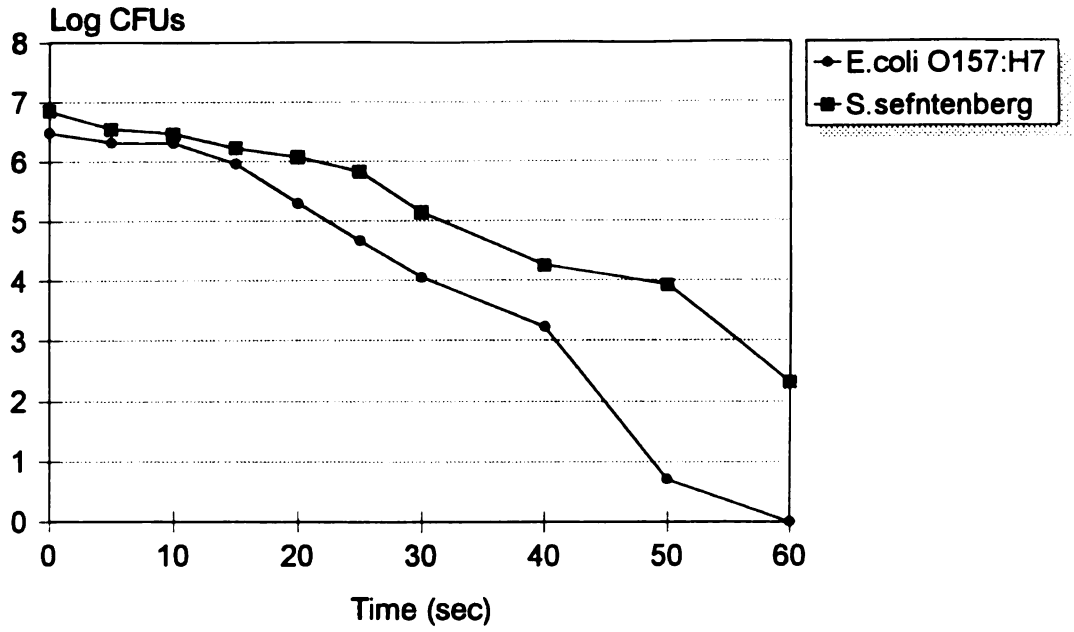


Figure 3.7. Survivor curves of *E.coli* O157:H7 and *S.senftenberg* in ground beef at 68°C.

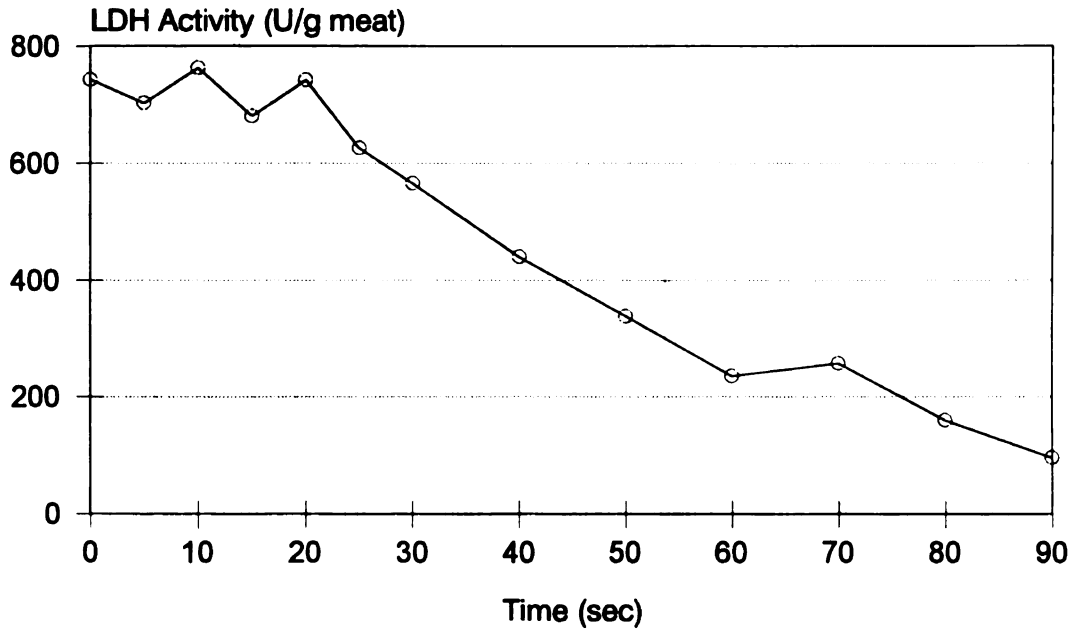


Figure 3.8. Thermal inactivation of LDH in ground beef at 68°C.

Table 3.3. Summary of D values and regression analysis for *E.coli* O157:H7 in ground beef.

T (°C)	D value (min)	Constant	R squared	X coefficient
53	46.10	6.86	0.96	-1.30
58	6.44	6.43	0.98	-0.16
63	0.43	7.47	0.93	-0.03
68	0.12	9.36	0.91	-0.16

Table 3.4. Summary of D values and regression analysis for *S.senftenberg* in ground beef.

T (°C)	D value (min)	Constant	R squared	X coefficient
53	53	5.69	0.90	-1.13
58	15.17	5.76	0.94	-0.07
63	2.08	5.23	0.81	-0.01
68	0.22	6.94	0.88	-0.07

Table 3.5. Summary of D values and regression analysis for LDH in ground beef.

T (°C)	D value (min)	Constant	R squared	X coefficient
58	543.48	2.88	0.55	-0.002
63	19.61	2.97	0.60	-0.001
68	1.40	3.15	0.90	-0.013

Table 3.6. Summary of z values and regression analysis for *E.coli* O157:H7, *S.senftenberg* and LDH in ground beef.

	z value (°C)	Constant	R squared	X coefficient
***E.coli* O157:H7**	5.60	10.77	0.98	-0.17
S.senftenberg	6.24	10.32	0.99	-0.16
LDH	3.86	17.22	0.99	-0.25

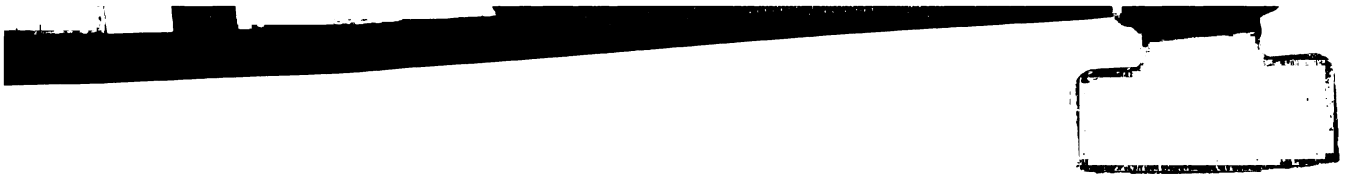


Table 3.7. Comparison of D and z values for *E.coli* O157:H7 and *S.senftenberg* in ground beef.

***E.coli* O157:H7**				***S.senftenberg***	
Doyle and Schoeni (1984)		**Line et al. (1991)**		**Goodfellow and Brown (1978)**	
T (°C)	**D value (min)**	**T (°C)**	**D value (min)**	**T (°C)**	**D value (min)**
54.4	39.8	51.7	78.2[a]- 80.1[b]	51.6	61[c]- 62[d]
57.2	4.5	57.2	4.1[a]- 4.0[b]	57.2	3.8[c]- 4.2[d]
62.8	0.4	62.8	0.30[a]- 0.22[b]	62.7	0.6[c]- 0.7[d]
z value (°C)	4.1	4.61[a] - 4.33[b]		**z value (°C)**	18

[a] PCA
[b] 2-h indole test
[c] Plate Count Agar overlayed with XL Agar base
[d] Most probable number technique

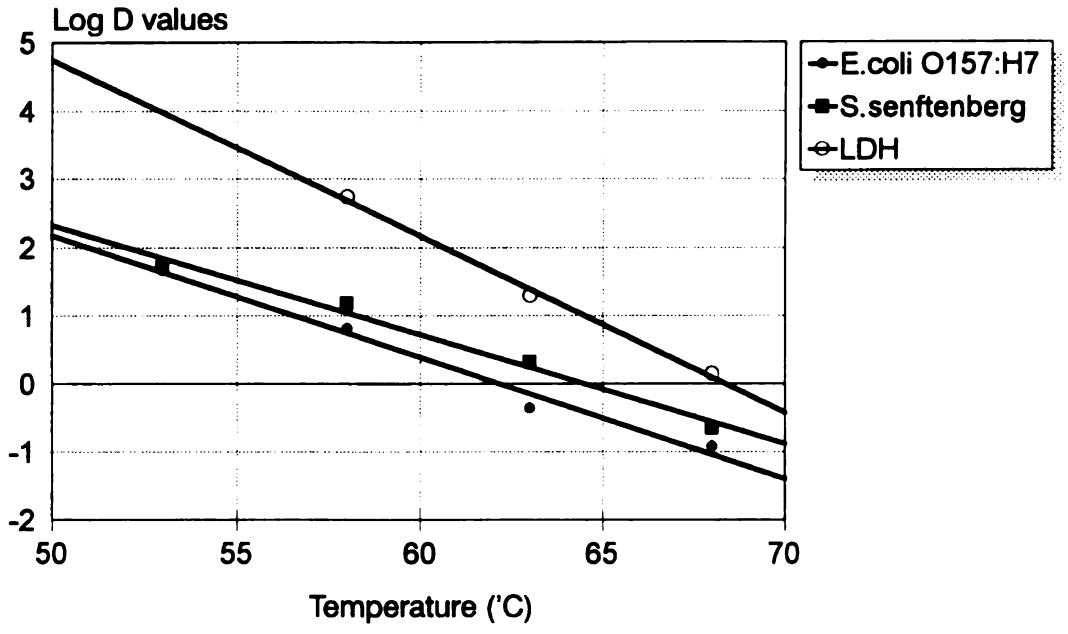
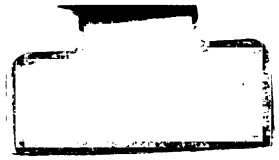


Figure 3.9. Thermal death time curves of *E.coli* O157:H7, *S.senftenberg* and LDH in ground beef.



respectively for each method. When compared to our results, Doyle and Schoeni obtained lower D values at all three temperatures. Line and co-workers obtained greater D values at 53° and 63°C and lower at 58°C. Neither one calculated D values above 65°C. In both studies the z value was 1-1.5°C lower than ours.

Discrepancies among results could be due to the use of different recovery methods. Doyle and Schoeni used Tryptic Soy agar capped with McConkey agar, and Line and co-workers used two different methods: Plate Count agar containing 1% pyruvate and 2-h indole test. In addition, while Line and co-workers used ground beef with a fat content (2%) similar to ours, Doyle and Schoeni used a much greater fat content (17-20%).

In a similar manner, Goodfellow and Brown (1978) calculated D values for *Salmonella* spp in ground beef (Table 3.7). The D values determined at 51.6°, 57.2° and 62.7°C were 61-62, 3.8-4.2, and 0.6-0.7 min, respectively. The z value was found to be 18°C. When compared to our results, our D_{53} was smaller while our D_{58} and D_{63} were greater than theirs. Again, discrepancies in the results could be due to use of different enumeration methods. Goodfellow and Brown used two different methods: for the first one they used Plate Count agar overlayed with XL Agar base supplemented with sodium thiosulfate and ferric ammonium sulfate, and for the second method they used the most probable number technique according to the Bacteriological Analytical Manual of the FDA. The authors did not mention the fat content of their meat.

Studies of LDH heat inactivation have been reported in bovine, porcine and poultry tissues. Collins et al. (1991a) observed a decrease in LDH activity in samples of beef top round (semimembranosus and abductor muscles) that had been heated to 54.5°C with a holding time of 121 min or to

60°, 62.8° or 65.6°C without a holding time. Enzymatic activity decreased by 26.6, 33.7, 77.1 and 96.5%, respectively. Similar trends were reported by the same authors in porcine muscle tissues (Collins et al.,1991b). Decreasing LDH activity with increasing temperature was observed when whole muscle hams were heated to 65°, 68° or 71°C. Samples heated to 65° and 68°C showed a significant reduction in LDH activity when compared to unheated samples, and almost undetectable levels were observed in samples heated to 71°C. Stalder et al. (1991) observed a reduction in LDH activity when extracts of bovine semimembranosus muscle were heated to 57°, 60°, 63° and 66°C. There was a large decrease in activity when internal temperature of samples reached 63°C and only marginal activity was detected at 66°C. In poultry, Wang et al. (1994) measured LDH activity in turkey breast rolls cooked to 68.3°, 69.7°, 70.9° and 72.1°C. Activity decreased from approximately 735 U/g meat in unheated samples to 22 U/g in samples heated to 68.3°C. Negligible activity (< 2.5 U/g meat) was detected above this temperature. Similarly, LDH activity in turkey thigh rolls decreased from 275 U/g meat to 44 U/g in samples processed to 72.2°C. Also, Desrocher (1994) observed a linear decrease in LDH activity in turkey hams with increasing processing temperatures. Initial activity in uncooked hams was 210 U/g meat and decreased to 39 U/g when cooked to 72.5°C.

While Collins and co-workers used semimembranosus and abductor muscles, Stalder and co-workers used semimembranosus muscle as the source of meat. Semitendinosus muscle was used in our study. Differences in LDH activity between bovine muscles have been described by Talmant et al. (1986) and Stalder et al. (1991). Muscles vary in their content of LDH isozymes according to their content and distribution of muscle fiber types. Therefore, different muscles show differences in LDH activity. Another

factor that can influence enzymatic activity of LDH is carcass maturity. Stalder et al. (1991) observed differences in LDH activity among A, B/C and D/E maturity groups. LDH activity was greatest in A and the lowest in D/E. Methodology employed in the experiment can also influence the results. Stalder et al. (1991) performed the heat inactivation in muscle slurries instead of whole muscle samples. Heating extracts results in higher inactivation at lower temperatures since the enzyme is more exposed to the heat stress. Collins and co-workers used whole muscle samples as we did, but the sample size as well as the TDT tubes, water bath setting and LDH extraction method were different from ours, which can have an impact on the results. In addition, after heating they stored the samples at 4°C for 3 weeks prior to analysis. Such a long period, even at refrigeration temperatures, can result in significant loss of enzymatic activity. Finally, the general approach in both Collins and Stalder studies was based on an endpoint temperature while we attempted to cover a temperature range over time.

3.5. CONCLUSIONS

LDH has already been recognized as a potential indicator of heat processing in beef and poultry products. We attempted to confirm its potential by comparing LDH thermal inactivation rate to those of *E.coli* O157:H7 and *S.senftenberg* using ground beef as a model. LDH was most heat resistant, whereas *E.coli* O157:H7 was the most heat sensitive. LDH had a greater D value than both microorganisms but smaller z value, meaning that the decrease in LDH enzymatic activity during heating was more pronounced than the reduction of survivors for both *E.coli* O157:H7 and *S.senftenberg*. Therefore, careful consideration of the time/temperature

relationship is of great importance when selecting an enzyme indicator to monitor the adequacy of previous heat treatments in meat and poultry products. In our case, LDH seems to be more suitable to be used as indicator of proper heat processing at the 65°-70°C range. At lower temperatures the slopes of the thermal death time curves of LDH, *E.coli* O157:H7 and *S.senftenberg* are so different that any possible relationship is difficult to be established.

4. PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST BOVINE MUSCLE LACTATE DEHYDROGENASE AND VERIFICATION OF ENDPOINT TEMPERATURE OF GROUND BEEF PATTIES BY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY

4.1. ABSTRACT

Monoclonal antibodies against bovine muscle LDH were produced and a sandwich enzyme-linked immunosorbent assay (ELISA) was developed using monoclonal and polyclonal antibodies. Ground beef patties containing 13.6% fat were cooked to internal temperatures of 62.8°, 65.6°, 68.3° and 71.1°C. Additionally, ground beef patties containing either 10.7 or 19.0% fat were cooked to an internal temperature of 68.3°C. Extracts of the ground beef patties were tested using the sandwich ELISA to detect differences in LDH concentration. Significant differences were found between patties cooked to 62.8°C and both uncooked patties and those cooked to 65.6°C. No differences were found among patties cooked to 65.6°, 68.3° and 71.1°C. Similarly, no significant differences in LDH content were found among patties containing different fat levels and cooked to 68.3°C.

4.2. INTRODUCTION

E.coli O157:H7 is an important cause of foodborne disease. Several outbreaks have been reported in the USA, Canada, United Kingdom, Mexico, China, Argentina and Belgium (Doyle, 1991). The severity of symptoms including death in some cases, have made it a real concern for food

processors, government regulatory agencies, and consumers. Most outbreaks have been associated with consumption of undercooked ground beef products.

Studies of thermal inactivation in ground beef have shown that *E.coli* O157:H7 is heat sensitive (Doyle and Schoeni, 1984; Line et al., 1991). Hence, one way to ensure its absence is to determine whether ground beef has achieved the proper endpoint cooking temperature at the center of the product. The Food and Drug Administration (FDA) recommends cooking ground beef patties by any of the following time/temperature protocols: 63°C (145°F)/3 min, 65°C (150°F)/1 min or 68°C (155°F)/15 sec (FDA, 1993). In addition, the US Department of Agriculture Food Safety Inspection Service (USDA-FSIS) requires official establishments that manufacture fully-cooked ground beef patties to cook patties using any of seven time/temperature schedules with holding time .

To determine proper endpoint temperature of beef products, the USDA currently employs a protein coagulation test (USDA, 1986), and a catalase enzyme test (USDA, 1989). These methods, however, are considered unreliable and do not provide enough margin of safety. There is a need for a more accurate and rapid assay which could be used for routine verification of proper heat processing. Several authors have suggested using residual enzyme activity as a way to detect the endpoint cooking temperature in beef products (Townsend and Davis, 1990; Stalder et al., 1991; Collins et al., 1991), but these assays are time consuming or require expensive equipment. On the other hand, the use of an enzyme indicator and further detection by immunoassays has been used successfully for the determination of endpoint cooking temperature in poultry products (Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993; Desrocher, 1994; Wang, 1994). The enzyme lactate

dehydrogenase (LDH) has already been shown to be a potential indicator of endpoint temperature of processing in beef (Collins et al., 1991; Stalder et al., 1991; Wang et al., 1994) and turkey products (Wang et al., 1992; Abouzied et al., 1993; Wang et al., 1993; Desrocher, 1994; Wang, 1994).

In recent studies we determined and compared the thermal inactivation of LDH with those of *E.coli* O157:H7 and *Salmonella senftenberg* (*S.senftenberg*) at four different temperatures (53°, 58°, 63° and 68°C). The results showed that LDH inactivation rate is slightly lower than that for both pathogens over the temperatures tested, confirming that this enzyme could be used as an indicator of proper thermal processing and, thus, ensure absence of pathogenic microorganisms in cooked ground beef products.

The purpose of this study was to develop a rapid and accurate assay to verify proper heat processing of ground beef patties. Monoclonal antibodies against bovine muscle were produced and a sandwich enzyme-linked immunosorbent assay (ELISA) was developed using the monoclonal antibodies and polyclonal antibodies that had been previously produced (Abouzied et al., 1994). The assay was used to detect changes in LDH concentration in patties containing the same amount of fat and cooked to four different endpoint temperatures, or patties with different fat content but cooked to the same internal temperature.

4.3. MATERIALS AND METHODS

4.3.1. Production of Monoclonal Antibodies

Two groups of 6-8 week-old female Balb\c mice (Charles River Laboratories, Wilmington, MA) were injected either subcutaneously or intraperitoneally (five mice each) with 75 μg of bovine muscle LDH (Sigma

Chemical Co., St. Louis, MO) in saline (0.8%) and mixed (1:1) with Freund's complete adjuvant (Sigma) for a total of 0.2 mL/mouse. Two booster injections were given at 2-week intervals as described above except that incomplete Freund's adjuvant (Sigma) was used. One week after the last injection, serum obtained from the retrobulbar plexus of each mice was tested by indirect ELISA to determine antibody titer, sensitivity and specificity. Three days before removal of the spleen for fusion, an intraperitoneal injection of bovine muscle LDH in saline solution was given to those mice with antisera showing the highest inhibition when tested by competitive ELISA (Abouzied et al, 1990).

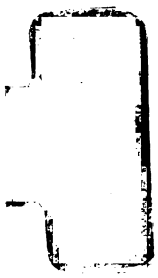
Monoclonal antibodies were produced following the procedure of Galfre and Milstein (1981) as modified by Abouzied et al. (1990). Mouse spleen cells ($1x10^8$) were fused with NS-1 myeloma cells ($1x10^7$) (P3/NS 1/1-Ag4-1, ATCC TIB 18) using 50% polyethylene glycol (Sigma) as the fusion agent. Following fusion, cells were suspended in Dulbecco's modified medium (Sigma) containing 20% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY), 1% NCTC medium (Gibco), 10 mM MEM sodium pyruvate solution (Gibco), and penicillin/streptomycin solution (100 U/mL) (Gibco). The cell suspension was then distributed into six 96-well flat bottom tissue culture plates (Corning Laboratory Science Co., Corning, NY). The plates were incubated at 37°C in a humid atmosphere containing 8% CO_2. After 24 h, half of the supernatant was removed from each well and replaced with an equal volume of hypoxanthine, aminopterin and thymidine (all three from Sigma) selective medium (HAT medium). Every three days the cells were fed exactly as above. After 2 weeks HAT medium was replaced by HT medium (same as HAT but without aminopterin). Those wells showing cell growth and color change were tested for antibody

production by competitive ELISA. Hybridomas showing continued production of anti-LDH antibodies were expanded and cloned twice by limiting dilution (Goding, 1980). Cloned cells were grown in HT medium containing 15% FBS and 15% macrophage-conditioned medium (Abouzied et al, 1990; Sugasawara et al., 1985).

Supernatants were collected and centrifuged to remove cells. Antibodies were purified by precipitation with 50% ammonium sulfate (Hebert et al., 1973), dialyzed for 3 days against 0.01M phosphate buffer saline (PBS, pH 7.2) at 4°C, aliquoted, and frozen.

4.3.2. Indirect ELISA

The indirect ELISA (Abouzied et al., 1990) was performed by coating microtiter plates (Immunolon-2 Removawells, Dynatech Laboratories Inc., Chantilly, VA) with 100 µL bovine LDH (10 µg/mL) in 0.1M carbonate buffer, pH 9.6, following incubation overnight at 4°C. Plates were washed 4 times with PBS containing 0.05% Tween-20 (Sigma) (PBS-T). Next, nonspecific binding sites were blocked by adding 300 µL of 1% ovoalbumin (Sigma) in PBS (OA-PBS) to each well and incubating at 37°C for 30 min. After washing 4 times with PBS-T, 50 µL of serially diluted serum were added to each well and incubated at 37°C for 1 h. After incubation, excess antibodies were removed by washing 4 times with PBS-T. To each well 100 µL of 1:500 goat-antimouse IgG peroxidase conjugate (Cappel Laboratories, West Chester, PA) in OA-PBS were added and incubated at 37°C for 30 min. After washing 8 times, peroxidase binding was determined by using 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma) (ABTS)-H_2O_2 substrate as described by Pestka et al. (1982). Absorbance at 405 nm was read using a Vmax Kinetic Microplate Reader (Model 1234, Molecular



Devices Corporation, Menlo Park, CA). The titer of each serum was arbitrarily determined as the dilution showing an optical density reading that was at least twice the reading of the non-immune serum at the same dilution.

The competitive indirect ELISA was identical as above except that after blocking of non-specific binding sites and washing, 50 μL of standard LDH were added to each well with 50 μL of the LDH antisera or 50 μL of cell culture supernatant.

4.3.3. Sandwich ELISA

The sandwich ELISA was performed by coating microtiter plates with 100 μL of monoclonal LDH antibodies in 0.1 M carbonate buffer, pH 9.6, (1:250) and drying overnight at 40°C in a forced air oven. After washing 4 times with PBS-T, nonspecific binding sites were blocked by adding 300 μL of 2% OA-PBS to each well and incubating at 37°C for 30 min. Plates were washed 4 times and 50 μL of serially diluted bovine muscle LDH or ground beef patties extracts were added to each well and incubated at 37°C for 30 min. After washing 4 more times, 50 μL/well of polyclonal antibody in 2% OA-PBS (1:500) were added. Following incubation for 30 min at 37°C and washing, 100 μL of 1:500 goat-antimouse IgG peroxidase conjugate in 2% OA-PBS were added to each well and incubated at 37°C for 30 min. After washing 8 times, bound peroxidase was determined by adding 100 μL K-Blue substrate (ELISA Technologies, Lexington, KY) to each well. Color development was stopped by addition of 100 μL/well of the Stopping reagent (ELISA Technologies). Absorbance was read at 650 nm.

4.3.4. Preparation of ground beef patties

Three eyes of the round (semitendinosus muscle) were trimmed, combined and used to prepare all patties. Muscles were ground twice through a 3.175 mm grinder plate in a Hobart grinder (Model 84181D, Hobart Mfg. Co., Troy, OH) and a sample was taken to analyze for fat content. The ground meat was separated into three batches. Ground beef kidney fat was added to achieve different levels of fat (10, 15 and 20%). Lean and fat were mixed in a chopper (Hobart) and reground twice through a 3.175 mm grinder plate. The ground meat was made into 113 g , 1.5 cm-thick patties using a burger press (Robinson Company Inc., Foley, Alabama). The patties were vacuum packaged and stored at 4°C until processed, within 5 days.

4.3.5. Cooking of ground beef patties

Patties were cooked using an electric frying pan (Faberware, Yonkers, NY) set at 190.6°C. Each patty was placed on the center of the pan and cooked on the first side for 3, 4 or 5 min, according to the internal temperature to be achieved. Patties were pressed for 1 min intervals with a spatula held in place with a 500 g weight. The patties were then turned and the temperature in the center of the patty was monitored using a template with a thermocouple connected to a control box (Model 660, Omega Engineering, Inc.). When the target temperature was reached the patties were immediately placed in an ice water bath. Total cooking time was recorded for each patty. Each experiment was repeated three times for each temperature and fat level. Three patties were cooked for each replicate for a total of 9 patties.

4.3.6. LDH extraction

Five grams from the core of each patty in a replicate were combined and extracted with 45 mL of cold phosphate buffer saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) using a Tissue Tearor™ at maximum speed for 1 min. The homogenate was centrifuged at 4340 x g for 20 min (Sorvall Superspeed RC2-B, Ivan Sorvall Inc, Norwalk, CT) and filtered through No. 1 Whatman paper. The filtrate was collected and stored at 4°C until analyzed, within 8 h.

4.3.7. Proximate analysis and determination of pH

Moisture, protein and fat composition of the ground beef were determined using AOAC (1990) methods 950.46B, 24.038 and 47.021, and 960.39, respectively. For determination of pH, 25 g ground beef were homogenized with 75 mL of distilled water in a Waring blender for 30 sec. The pH of the homogenate was measured using a Corning 145 pH meter (Fisher Scientific, Itasca, IL).

4.3.8. Statistical analysis

The analysis of variance (ANOVA) was calculated using a randomized complete block design. Mean LDH concentrations in ground beef patties were compared using Student-Neuman- Keul's Multiple range test of the MSTAT statistical program (MSTAT, 1994) with the mean square error term at the 5% level of probability.

4.4. RESULTS AND DISCUSSION

4.4.1. Production of monoclonal antibodies

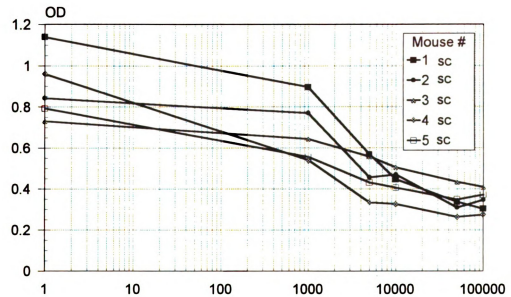
As a general rule, mice injected subcutaneously with bovine muscle LDH showed higher serum titers than those injected intraperitoneally (data not shown). In addition, when tested by competitive indirect ELISA, sera of mice injected subcutaneously showed higher inhibition than those injected intraperitoneally (Figure 4.1. and 4.2.).

A total of 360 wells were seeded with fused cells from the spleen of the mouse with the highest titer and NS-1 myeloma cells, but only one produced antibodies against LDH. A second fusion was performed using two mice which resulted in two more positive hybridomas.

The three antibody-producing hybridomas, designated as 5E2, 4C5 and 5F8, were further expanded and cloned. Eight stabilized lines were obtained which showed high inhibition when tested by indirect competitive ELISA. The cell lines were designated as:

- #3 B131-B6E6-11F6
- #5 B131-B6E6-11F10
- #7 B131-B6E6-11D3
- #9 B131-B6E6-11D9
- #15 A731-G3A2-11G5
- #16 A731-G3A2-1D10
- #17 A731-G3A2-11D9
- #24 A731-G3A2-12C4

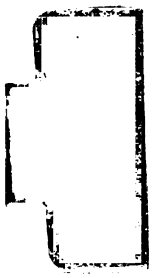
A sandwich ELISA was developed with monoclonal antibodies (B131-B6E6-11D3) as the capture antibodies and polyclonal antibodies as the detector antibodies. Detection of LDH by sandwich ELISA is shown in Figure 4.3.

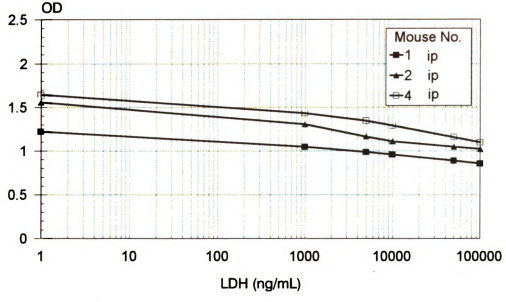


Sera dilutions are:

1) 1: 10,000
2) 1: 12,000
3) 1: 10,000
4) 1: 7,000
5) 1: 8,000

Figure 4.1. Detection of bovine muscle LDH by competitive indirect ELISA using sera of mice injected subcutaneously (sc).





Sera dilutions are:
1) 1: 8,000
2) 1: 10,000
4) 1: 8,000

Figure 4.2. Detection of bovine muscle LDH by competitive indirect ELISA using sera of mice injected intraperitoneally (ip).

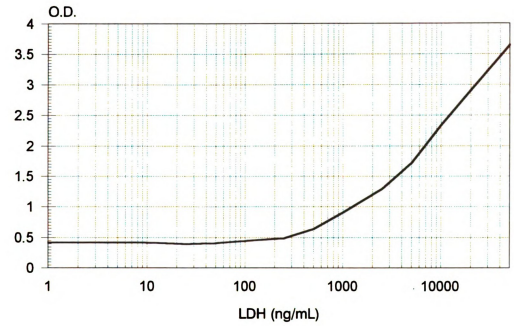


Figure 4.3. Detection of bovine muscle LDH by sandwich ELISA: monoclonal antibodies (1: 250) as capture antibodies and polyclonal antibodies (1: 500) as detector antibodies.

4.4.2. Preparation and cooking of ground beef patties

Three different batches of ground beef patties containing three different levels of fat were prepared. Results of proximate analysis and determination of pH for the ground beef patties are shown in Table 4.1. Moisture ranged from 64.3 to 67.6% and protein from 15.7 to 19.6%. Fat levels achieved were 10.7, 13.6 and 19.0%. The pH ranged from 6.42 to 6.43.

Cooking schedules are shown in Table 4.2. Nine patties were cooked for each temperature and fat level. Ground beef patties containing 13.6% fat were cooked to internal temperatures of 62.8°, 65.6°, 68.3° and 71.1°C. Patties containing either 10.7 or 19.0% fat were cooked to an internal temperature of 68.3°C. Total cooking time ranged from 9 min in patties cooked to 62.8°C to 18 min in patties cooked to 71.1°C. Great variation was observed in terms of cooking rate time among patties in a triplicate. The maximum variation in total time was observed in patties cooked to 68.3°C independently of the fat content.

LDH was extracted using a Tissue Tearor™. This method of extraction was more efficient than homogenization in a blender, the common method of extraction employed in similar studies (Collins et al., 1991; Stalder et al., 1991; Wang and Smith, 1994). As a consequence, higher concentrations of LDH were observed in both cooked and uncooked patties.

Extracts from the patties were tested using a sandwich ELISA. LDH content in extracts from raw patties was 13.1 mg/g, 10.4 mg/g and 8.18 mg/g meat, corresponding to fat concentrations of 10.7, 13.6 and 19.0%, respectively. The LDH content decreased significantly ($p < 0.05$) in patties cooked to 62.8°C (114 μg/g) when compared to the uncooked patties (10.4 mg/g). Significant differences ($p < 0.05$) were also observed between patties cooked to 62.8°C and those cooked to 65.6°(54 μg/g). No

Table 4.1. Proximate analysis of ground beef patties[a].

Moisture (%)	Protein (%)	Fat (%)	pH
67.6	19.6	10.7	6.42
66.1	17.6	13.6	6.42
64.3	15.7	19.	6.43

[a] Average of three samples.

Table 4.2. Summary of ground beef patties cooking.

Internal Temperature (°C)	Fat Level (%)	First Side Cooking Time (min)	Total Cooking Time[a] (min)
62.8	13.6	3	9.69 ± 1.4
65.6	13.6	4	12.04 ± 2.5
68.3	10.7	4	14.1 ± 4.62
68.3	13.6	4	16.21 ± 3.7
68.3	19.0	4	16.31 ± 4.51
71.1	13.6	5	18.55 ± 3.2

[a]Expressed as mean ± standard deviation.

significant differences ($p > 0.05$), however, were found among patties cooked to 65.6° (54 μg/g), 68.3° (57.2 μg/g) and 71.1°C (21.8 μg/g) (Figure 4.4.).

In patties containing different levels of fat and cooked to 68.3°C, average LDH content was 57.5 μg/g and 21.9μg/g, corresponding to 10.7 and 19.0% fat contents. No significant differences ($p > 0.05$) were found among these values and the patties containing 13.6% fat (Figure 4.5.).

In a parallel experiment, the same ground beef extracts were tested by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and LDH enzymatic assay. Similar results to those of sandwich ELISA were obtained by these three methods (data not shown).

Similar studies have been done in poultry products with successful results. Antibodies against turkey LDH could detect differences in LDH content of extracts between turkey thigh rolls that had been cooked to 71.1° and 72.2°C (Wang, 1994). The same antibodies could detect differences in LDH content of extracts from turkey breast rolls that had been cooked to 69.7°C and 70.9°C (Wang et al., 1993).

That the bovine muscle sandwich ELISA did not work in this experiment does not mean that the approach was a complete failure. The assay could detect different concentrations of LDH even though the detection limit was not as high as expected. Also, the variation among patties within a replicate suggests that the same experiment should be repeated with patties cooked under more controlled conditions.

5. CONCLUSIONS

Bovine muscle LDH had poor antigenic properties when injected into mice for production of monoclonal antibodies. A very low percentage

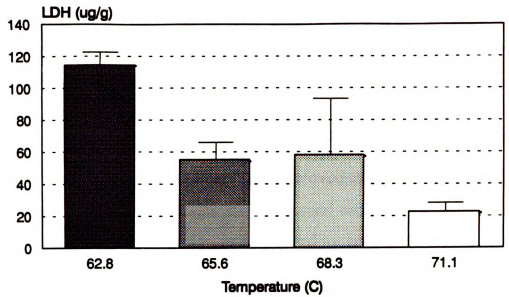


Figure 4.4. Concentration of LDH in ground beef patties cooked to different internal temperatures.

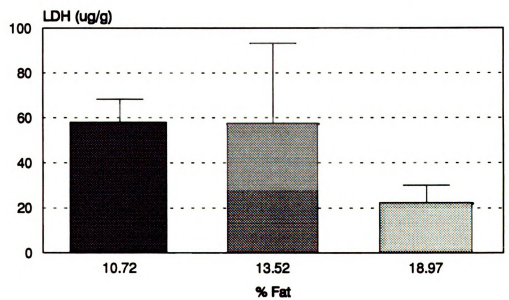


Figure 4.5. Concentration of LDH in ground beef patties with different fat content cooked to 68.3°C.

(0.004%) of anti-LDH antibodies-producing hybridomas was obtained after two fusions. A sandwich ELISA using monoclonal and polyclonal antibodies against bovine LDH has potential for determination of LDH concentration in beef products. If a relationship between LDH concentration and endpoint temperature can be established, this method could be successfully used for verification of proper thermal processing of beef products.

Cooking of ground beef patties proved to be very inconsistent. High variation in temperature rise as well as total cooking time among patties cooked under exact conditions accounted for high variations in the final product. This factor interferes with the accurate detection of any protein marker. Future research should include the development of a more accurate and consistent method of cooking. Once a controlled cooking method is established, the LDH sandwich ELISA should be tested further.

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APPENDICES

APPENDIX A

Appendix A

Comparison of three media for enumeration of *Escherichia coli* O157:H7

Table A.1. Mean log CFU/mL for regular *E.coli* O157:H7 culture[a].

Media	1st Run	2nd Run	3rd Run
Coliform Count Plates	8.87±0.04	8.89±0.05	8.94±0.06
***E.coli* Count Plates**	8.88±0.06	8.88±0.06	8.93±0.05
MC-PCA	8.77±0.06	8.82±0.06	8.88±0.18

[a] Values expressed as mean ± SD.

Table A.2. Mean log CFU/mL for heat-shocked *E.coli* O157:H7 culture[a].

Media	1st Run	2nd Run	3rd Run
Coliform Count Plates	4.88±0.05	4.76±0.06	4.44±0.1
***E.coli* Count Plates**	4.87±0.03	4.78±0.02	4.45±0.09
MC-PCA	4.63±0.15	4.68±0.06	4.54±0.04

[a] Values expressed as mean ± SD.

APPENDIX B

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Appendix B

Thermal inactivation of *Escherichia coli* O157:H7 as compared to that of the enzyme lactate dehydrogenase and *Salmonella senftenberg*

E.coli O157:H7 at 53°C

1) 1st Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	1.19E+06	1.02E+06	6.08	6.01	6.04
0	9.10E+06	9.20E+06	6.96	6.96	6.96
0.5	3.30E+06	2.80E+06	6.52	6.45	6.48
1	5.10E+05	6.40E+05	5.71	5.81	5.76
1.5	3.30E+05	2.50E+05	5.52	5.40	5.46
2	4.70E+04	5.10E+04	4.67	4.71	4.69
3	5.40E+02	3.00E+02	2.73	2.48	2.60
4	3.00E+01	2.00E+01	1.48	1.30	1.39
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

2) 2nd Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	1.19E+06	1.02E+06	6.08	6.01	6.04
0	7.90E+06	5.00E+06	6.90	6.70	6.80
0.5	3.00E+06	3.60E+06	6.48	6.56	6.52
1	2.90E+06	2.40E+06	6.46	6.38	6.42
1.5	7.00E+05	6.90E+05	5.85	5.84	5.84
2	2.60E+05	3.10E+05	5.41	5.49	5.45
3	1.07E+04	9.90E+03	4.03	4.00	4.01
4	0.00E+00	0.00E+00	0.00	0.00	0.00
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

3) 3rd Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	6.80E+06	6.80E+06	6.83	6.83	6.83
0	2.40E+06	2.90E+06	6.38	6.46	6.42
0.5	1.00E+06	1.06E+06	6.00	6.03	6.01
1	1.76E+05	1.78E+05	5.25	5.25	5.25
1.5	1.40E+04	5.00E+03	4.15	3.70	3.92
2	3.00E+03	3.90E+03	3.48	3.59	3.53
3	5.00E+01	6.00E+01	1.70	1.78	1.74
4	0.00E+00	0.00E+00	0.00	0.00	0.00
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

S.eefternberg at 53°C

1) 1st Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	1.01E+07	1.19E+07	7.00	7.08	7.04
0	7.90E+06	7.90E+06	6.90	6.90	6.90
0.5	3.10E+05	3.20E+05	5.49	5.51	5.50
1	1.20E+04	2.40E+04	4.08	4.38	4.23
1.5	8.00E+03	9.00E+03	3.90	3.95	3.93
2	2.00E+02	3.00E+02	2.30	2.48	2.39
3	3.00E+01	2.00E+01	1.48	1.30	1.39
4	1.00E+01	1.00E+01	1.00	1.00	1.00
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

2) 2nd Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	1.41E+07	1.32E+07	7.15	7.12	7.13
0	1.84E+07	1.82E+07	7.26	7.26	7.26
0.5	1.60E+05	1.80E+05	5.20	5.26	5.23
1	1.50E+04	1.60E+04	4.18	4.20	4.19
1.5	2.00E+03	1.00E+03	3.30	3.00	3.15
2	3.00E+02	3.00E+02	2.48	2.48	2.48
3	2.70E+02	3.40E+02	2.43	2.53	2.48
4	1.00E+01	0.00E+00	1.00	0.00	0.50
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

3) 3rd Run

Time (hrs)	1	2	Log 1	Log 2	Average
Raw	1.07E+07	1.19E+07	7.03	7.08	7.05
0	9.30E+06	9.60E+06	6.97	6.98	6.98
0.5	3.50E+05	2.30E+05	5.54	5.36	5.45
1	2.80E+04	3.40E+04	4.45	4.53	4.49
1.5	2.00E+03	2.00E+03	3.30	3.30	3.30
2	3.00E+02	6.00E+02	2.48	2.78	2.63
3	1.40E+02	1.30E+02	2.15	2.11	2.13
4	0.00E+00	0.00E+00	0.00	0.00	0.00
5	0.00E+00	0.00E+00	0.00	0.00	0.00
6	0.00E+00	0.00E+00	0.00	0.00	0.00

LDH 53'c

1) 1st Run

Time (hrs)	1	2	Log 1	Log 2	Average
0	MISSING	MISSING			0
0.5	6.85E+02	6.75E+02	2.84	2.83	2.83
1	8.39E+02	8.38E+02	2.92	2.92	2.92
1.5	9.86E+02	7.21E+02	2.99	2.86	2.93
2	8.93E+02	8.81E+02	2.95	2.95	2.95
3	1.05E+03	1.01E+03	3.02	3.00	3.01
4	8.23E+02	7.91E+02	2.92	2.90	2.91
5	6.75E+02	7.99E+02	2.83	2.90	2.87
6	9.11E+02	8.88E+02	2.96	2.95	2.95

2) 2nd Run

Time (hrs)	1	2	Log 1	Log 2	Average
0	4.72E+02	5.12E+02	2.67	2.71	2.69
0.5	6.70E+02	7.16E+02	2.83	2.86	2.84
1	4.80E+02	4.38E+02	2.68	2.64	2.66
1.5	3.29E+02	2.96E+02	2.52	2.47	2.49
2	6.90E+02	7.31E+02	2.84	2.86	2.85
3	9.78E+02	8.21E+02	2.99	2.91	2.95
4	7.57E+02	5.89E+02	2.88	2.77	2.82
5	5.58E+02	5.71E+02	2.75	2.76	2.75
6	2.74E+02	2.44E+02	2.44	2.39	2.41

3) 3rd Run

Time (hrs)	1	2	Log 1	Log 2	Average
0	3.24E+02	4.00E+02	2.51	2.60	2.56
0.5	4.95E+02	4.78E+02	2.69	2.68	2.69
1	5.28E+02	5.30E+02	2.72	2.72	2.72
1.5	3.66E+02	3.49E+02	2.56	2.54	2.55
2	4.87E+02	5.05E+02	2.69	2.70	2.70
3	3.34E+02	3.43E+02	2.52	2.53	2.53
4	4.74E+02	4.56E+02	2.68	2.66	2.67
5	4.80E+02	4.51E+02	2.68	2.65	2.67
6	MISSING	MISSING			

E. coli O157:H7 at 58°C

1)1st RUN

Time (min)	1	2	Log 1	Log 2	Average
0	6.50E+06	6.60E+06	6.81	6.82	6.82
2	1.40E+06	1.80E+06	6.15	6.26	6.20
4	1.50E+06	8.70E+05	6.18	5.94	6.06
8	7.60E+04	7.20E+04	4.88	4.86	4.87
10	3.60E+04	2.80E+04	4.56	4.45	4.50
15	1.53E+03	1.85E+03	3.18	3.27	3.23
20	3.00E+02	4.80E+02	2.48	2.68	2.58
30	1.00E+01	0.00E+00	1.00	0.00	0.50
35	MISSING	MISSING			0.00
45	0.00E+00	0.00E+00	0.00	0.00	0.00

2)2nd RUN

Time (min)	1	2	Log 1	Log 2	Average
0	3.30E+06	3.30E+06	6.52	6.52	6.52
2	1.69E+06	1.56E+06	6.23	6.19	6.21
4	1.44E+06	1.01E+06	6.16	6.00	6.08
8	1.86E+05	2.00E+05	5.27	5.30	5.29
10	4.90E+04	5.10E+04	4.69	4.71	4.70
15	6.60E+03	7.40E+03	3.82	3.87	3.84
20	8.60E+03	7.40E+03	3.93	3.87	3.90
30	0.00E+00	2.00E+01	0.00	1.30	0.65
35	0.00E+00	0.00E+00	0.00	0.00	0.00
45	0.00E+00	0.00E+00	0.00	0.00	0.00

3)3rd RUN

Time (min)	1	2	Log 1	Log 2	Average
0	6.50E+06	5.80E+06	6.81	6.76	6.79
2	2.50E+06	2.30E+06	6.40	6.36	6.38
4	1.91E+06	1.90E+06	6.28	6.28	6.28
8	3.80E+05	2.80E+05	5.58	5.45	5.51
10	1.11E+05	1.08E+05	5.05	5.03	5.04
15	4.70E+03	3.10E+03	3.67	3.49	3.58
20	1.25E+03	1.12E+03	3.10	3.05	3.07
30	2.80E+02	3.90E+02	2.45	2.59	2.52
35	2.00E+02	1.20E+02	2.30	2.08	2.19
45	0.00E+00	0.00E+00	0.00	0.00	0.00

S.eoftemberg at 58°C

1)1st RUN

Time (min)	1	2	Log 1	Log 2	Average
0	1.91E+06	1.76E+06	6.28	6.25	6.26
10	1.02E+05	1.29E+05	5.01	5.11	5.06
20	2.50E+04	2.50E+04	4.40	4.40	4.40
30	4.50E+03	2.60E+03	3.65	3.41	3.53
40	4.00E+02	1.00E+02	2.60	2.00	2.30
50	5.20E+02	6.80E+02	2.72	2.83	2.77
60	2.10E+02	1.90E+02	2.32	2.28	2.30
70	8.00E+01	8.00E+01	1.90	1.90	1.90
80	0.00E+00	0.00E+00	0.00	0.00	0.00
90	0.00E+00	0.00E+00	0.00	0.00	0.00

2)2nd RUN

Time (min)	1	2	Log 1	Log 2	Average
0	2.60E+06	2.50E+06	6.41	6.40	6.41
10	1.71E+05	1.52E+05	5.23	5.18	5.21
20	1.30E+04	2.10E+04	4.11	4.32	4.22
30	2.40E+03	4.20E+03	3.38	3.62	3.50
40	1.00E+02	3.00E+02	2.00	2.48	2.24
50	6.00E+02	4.90E+02	2.78	2.69	2.73
60	2.30E+02	8.00E+01	2.36	1.90	2.13
70	8.00E+01	2.00E+01	1.90	1.30	1.60
80	0.00E+00	2.00E+01	0.00	1.30	0.65
90	0.00E+00	0.00E+00	0.00	0.00	0.00

3)3rd RUN

Time (min)	1	2	Log 1	Log 2	Average
0	2.60E+06	3.70E+06	6.41	6.57	6.49
10	5.50E+05	5.30E+05	5.74	5.72	5.73
20	3.40E+04	3.40E+04	4.53	4.53	4.53
30	1.00E+02	1.00E+02	2.00	2.00	2.00
40	1.00E+02	1.00E+02	2.00	2.00	2.00
50	5.00E+02	6.10E+02	2.70	2.79	2.74
60	9.00E+01	8.00E+01	1.95	1.90	1.93
70	0.00E+00	0.00E+00	0.00	0.00	0.00
80	1.00E+01	2.00E+01	1.00	1.30	1.15
90	0.00E+00	0.00E+00	0.00	0.00	0.00

LDH 58°C

1)1st Run

Time(min)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	8.74E+02	8.74E+02	2.94	2.94	2.94
2	1.20E+03	1.20E+03	3.08	3.08	3.08
4	1.11E+03	1.11E+03	3.04	3.04	3.04
8	9.81E+02	9.81E+02	2.99	2.99	2.99
10	6.63E+02	6.63E+02	2.82	2.82	2.82
15	5.52E+02	5.82E+02	2.74	2.76	2.75
20	6.42E+02	5.87E+02	2.81	2.77	2.79
30	6.90E+02	6.90E+02	2.84	2.84	2.84
35	7.00E+02	7.00E+02	2.84	2.84	2.84
45	6.95E+02	6.95E+02	2.84	2.84	2.84

2) 2nd Run

Time(min)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	7.59E+02	6.59E+02	2.88	2.82	2.85
2	5.71E+02	7.16E+02	2.76	2.86	2.81
4	6.61E+02	6.05E+02	2.82	2.78	2.80
8	7.72E+02	7.72E+02	2.89	2.89	2.89
10	7.37E+02	6.97E+02	2.87	2.84	2.86
15	7.12E+02	6.32E+02	2.85	2.80	2.83
20	7.17E+02	7.42E+02	2.86	2.87	2.86
30	6.32E+02	6.27E+02	2.80	2.80	2.80
35	5.12E+02	5.17E+02	2.71	2.71	2.71
45	6.42E+02	6.32E+02	2.81	2.80	2.80

3) 3rd Run

Time(min)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	6.32E+02	5.92E+02	2.80	2.77	2.79
2	5.77E+02	5.60E+02	2.76	2.75	2.75
4	7.29E+02	6.22E+02	2.86	2.79	2.83
8	7.10E+02	6.02E+02	2.85	2.78	2.82
10	6.30E+02	7.75E+02	2.80	2.89	2.84
15	6.46E+02	6.16E+02	2.81	2.79	2.80
20	8.69E+02	7.05E+02	2.94	2.85	2.89
30	7.13E+02	7.72E+02	2.85	2.89	2.87
35	7.16E+02	7.52E+02	2.85	2.88	2.87
45	5.57E+02	5.47E+02	2.75	2.74	2.74

E.coli O157:H7 at 63°C

1)1st RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	7.30E+06	9.20E+06	6.86	6.96	6.91
60	8.30E+06	9.10E+06	6.92	6.96	6.94
75	4.10E+06	4.00E+06	6.61	6.60	6.61
90	1.57E+06	1.72E+06	6.20	6.24	6.22
100	6.00E+05	5.90E+05	5.78	5.77	5.77
110	3.20E+05	5.00E+05	5.51	5.70	5.60
120	5.50E+05	4.50E+05	5.74	5.65	5.70
130	1.19E+05	1.15E+05	5.08	5.06	5.07
140	1.20E+03	3.20E+03	3.08	3.51	3.29
150	5.90E+04	5.60E+04	4.77	4.75	4.76
160	99	99	2.00	2.00	2.00
170	0.00E+00	0.00E+00	0.00	0.00	0.00
180	0.00E+00	0.00E+00	0.00	0.00	0.00
210	MISSING	MISSING			0.00
240	0.00E+00	0.00E+00	0.00	0.00	0.00

2)2nd RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	7.30E+06	9.20E+06	6.86	6.96	6.91
60	5.30E+06	4.30E+06	6.72	6.63	6.68
75	2.70E+06	2.50E+06	6.43	6.40	6.41
100	1.56E+06	1.73E+06	6.19	6.24	6.22
110	8.40E+05	7.80E+05	5.92	5.89	5.91
120	2.11E+05	2.59E+05	5.32	5.41	5.37
130	1.58E+05	1.34E+05	5.20	5.13	5.16
140	3.76E+04	1.90E+04	4.58	4.28	4.43
150	4.60E+04	2.60E+04	4.66	4.41	4.54
160	7.70E+03	1.00E+04	3.89	4.00	3.94
170	4.30E+02	5.50E+02	2.63	2.74	2.69
180	1.09E+03	1.06E+03	3.04	3.03	3.03
210	0.00E+00	0.00E+00	0.00	0.00	0.00
240	0.00E+00	0.00E+00	0.00	0.00	0.00

3)3rd RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	7.30E+06	9.20E+06	6.86	6.96	6.91
60	4.40E+06	4.70E+06	6.64	6.67	6.66
75	4.80E+06	2.70E+06	6.68	6.43	6.56
90	1.18E+06	1.62E+06	6.07	6.21	6.14
100	9.20E+05	6.40E+05	5.96	5.81	5.88
110	6.70E+05	7.40E+05	5.83	5.87	5.85
120	1.23E+05	1.23E+05	5.09	5.09	5.09
130	8.80E+04	3.50E+04	4.94	4.54	4.74
140	1.65E+04	1.70E+04	4.22	4.23	4.22
150	7.50E+03	5.50E+03	3.88	3.74	3.81
160	9.00E+02	8.00E+02	2.95	2.90	2.93
170	1.31E+03	1.82E+03	3.12	3.26	3.19
180	30	20	1.48	1.30	1.39
210	2.40E+02	1.80E+02	2.38	2.26	2.32
240	9.00E+00	9.00E+00	0.95	0.95	0.95

S.eefternberg at 63°C

1)1st RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	3.80E+06	3.70E+06	6.58	6.57	6.57
60	2.50E+06	3.20E+06	6.40	6.51	6.45
120	2.40E+05	3.70E+05	5.38	5.57	5.47
150	6.20E+04	5.90E+04	4.79	4.77	4.78
180	1.66E+04	1.37E+04	4.22	4.14	4.18
240	2.10E+03	9.00E+02	3.32	2.95	3.14
270	6.00E+02	5.00E+02	2.78	2.70	2.74
300	1.00E+02	0.00E+00	2.00	0.00	1.00
360	2.40E+02	3.40E+02	2.38	2.53	2.46
420	9.00E+01	7.00E+01	1.95	1.85	1.90
480	1.00E+01	1.00E+01	1.00	1.00	1.00
600	1.00E+01	2.00E+01	1.00	1.30	1.15

2)2nd RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	3.80E+06	3.70E+06	6.58	6.57	6.57
60	3.10E+06	2.70E+06	6.49	6.43	6.46
120	3.90E+05	3.20E+05	5.59	5.51	5.55
150	1.08E+05	1.15E+05	5.03	5.06	5.05
180	1.15E+04	1.12E+04	4.06	4.05	4.05
240	1.10E+03	6.00E+02	3.04	2.78	2.91
270	7.00E+02	3.00E+02	2.85	2.48	2.66
300	2.00E+02	3.00E+02	2.30	2.48	2.39
360	1.10E+02	8.00E+01	2.04	1.90	1.97
420	2.00E+01	2.00E+01	1.30	1.30	1.30
480	1.00E+02	6.00E+01	2.00	1.78	1.89
600	1.00E+01	2.00E+01	1.00	1.30	1.15

3)3rd RUN

Time (sec)	1	2	Log 1	Log 2	Average
0	3.80E+06	3.70E+06	6.58	6.57	6.57
60	1.78E+06	1.93E+06	6.25	6.29	6.27
120	2.50E+05	1.60E+05	5.40	5.20	5.30
150	1.39E+05	1.26E+05	5.14	5.10	5.12
180	1.89E+04	1.85E+04	4.28	4.27	4.27
240	1.10E+03	5.00E+02	3.04	2.70	2.87
270	9.00E+02	4.00E+02	2.95	2.60	2.78
300	5.00E+02	6.00E+02	2.70	2.78	2.74
360	1.00E+02	1.10E+02	2.00	2.04	2.02
420	4.00E+01	2.00E+01	1.60	1.30	1.45
480	1.00E+01	2.00E+01	1.00	1.30	1.15
600	1.00E+01	2.00E+01	1.00	1.30	1.15

LDH 63'C

1) 1st Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	1.15E+03	1.18E+03	3.06	3.07	3.07
60	9.86E+02	9.95E+02	2.99	3.00	3.00
75	9.03E+02	8.25E+02	2.96	2.92	2.94
90	1.33E+03	1.01E+03	3.12	3.00	3.06
100	7.78E+02	8.29E+02	2.89	2.92	2.90
110	6.20E+02	6.02E+02	2.79	2.78	2.79
120	6.04E+02	4.54E+02	2.78	2.66	2.72
130	6.27E+02	6.38E+02	2.80	2.80	2.80
140	5.99E+02	5.80E+02	2.78	2.76	2.77
150	5.20E+02	5.99E+02	2.72	2.78	2.75
160	6.97E+02	7.18E+02	2.84	2.86	2.85
170	7.31E+02	7.07E+02	2.86	2.85	2.86
180	6.16E+02	5.81E+02	2.79	2.76	2.78
210	6.43E+02	6.07E+02	2.81	2.78	2.80
240	6.06E+02	5.38E+02	2.78	2.73	2.76

2) 2nd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	1.00E+03	9.95E+02	3.00	3.00	3.00
60	7.98E+02	7.90E+02	2.90	2.90	2.90
75	7.93E+02	7.93E+02	2.90	2.90	2.90
90	7.62E+02	7.72E+02	2.88	2.89	2.88
100	7.46E+02	7.42E+02	2.87	2.87	2.87
110	7.45E+02	7.26E+02	2.87	2.86	2.87
120	7.72E+02	8.02E+02	2.89	2.90	2.90
130	8.13E+02	7.39E+02	2.91	2.87	2.89
140	8.13E+02	7.83E+02	2.91	2.89	2.90
150	7.83E+02	7.97E+02	2.89	2.90	2.90
160	5.91E+02	6.50E+02	2.77	2.81	2.79
170	7.75E+02	7.41E+02	2.89	2.87	2.88
180	7.69E+02	7.56E+02	2.89	2.88	2.88
210	5.99E+02	5.48E+02	2.78	2.74	2.76
240	6.17E+02	6.54E+02	2.79	2.82	2.80

3) 3rd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	7.47E+02	7.54E+02	2.87	2.88	2.88
60	7.58E+02	7.29E+02	2.88	2.86	2.87
75	7.06E+02	7.75E+02	2.85	2.89	2.87
90	7.46E+02	6.78E+02	2.87	2.83	2.85
100	6.21E+02	6.00E+02	2.79	2.78	2.79
110	9.61E+02	9.61E+02	2.98	2.98	2.98
120	9.45E+02	9.45E+02	2.98	2.98	2.98
130	7.98E+02	8.30E+02	2.90	2.92	2.91
140	6.39E+02	6.13E+02	2.81	2.79	2.80
150	6.24E+02	6.78E+02	2.80	2.83	2.81
160	6.27E+02	6.42E+02	2.80	2.81	2.80
170	6.83E+02	5.95E+02	2.83	2.77	2.80
180	4.94E+02	4.81E+02	2.69	2.68	2.69
210	5.20E+02	4.96E+02	2.72	2.70	2.71
240	5.34E+02	5.36E+02	2.73	2.73	2.73

E.coli O157:H7 at 68°C

1) 1st Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	3.20E+06	2.90E+06	6.51	6.46	6.48
0	1.67E+06	1.47E+06	6.22	6.17	6.20
5	2.70E+06	2.80E+06	6.43	6.45	6.44
10	1.85E+06	1.82E+06	6.27	6.26	6.26
15	1.06E+06	1.07E+06	6.03	6.03	6.03
20	1.86E+05	1.93E+05	5.27	5.29	5.28
25	4.40E+04	6.00E+04	4.64	4.78	4.71
30	4.60E+03	6.80E+03	3.66	3.83	3.75
40	1.85E+03	1.50E+03	3.27	3.18	3.22
50	1.00E+02	1.70E+02	2.00	2.23	2.12
60	0.00E+00	0.00E+00	0.00	0.00	0.00

2) 2nd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	3.20E+06	2.90E+06	6.51	6.46	6.48
0	4.30E+06	4.60E+06	6.63	6.66	6.65
5	1.78E+06	1.53E+06	6.25	6.18	6.22
10	2.15E+06	2.28E+06	6.33	6.36	6.35
15	1.00E+06	1.14E+06	6.00	6.06	6.03
20	1.66E+05	1.90E+05	5.22	5.28	5.25
25	3.05E+04	2.59E+04	4.48	4.41	4.45
30	1.69E+04	1.63E+04	4.23	4.21	4.22
40	MISSING	MISSING			0.00
50	0.00E+00	0.00E+00	0.00	0.00	0.00
60	0.00E+00	0.00E+00	0.00	0.00	0.00

3) 3rd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	3.20E+06	2.90E+06	6.51	6.46	6.48
0	1.35E+06	1.28E+06	6.13	6.11	6.12
5	1.80E+06	2.12E+06	6.26	6.33	6.29
10	9.80E+05	1.18E+06	5.99	6.07	6.03
15	7.40E+05	6.30E+05	5.87	5.80	5.83
20	2.20E+05	2.40E+05	5.34	5.38	5.36
25	7.90E+04	6.20E+04	4.90	4.79	4.85
30	1.51E+04	1.48E+04	4.18	4.17	4.17
40	MISSING	MISSING			0.00
50	0.00E+00	0.00E+00	0.00	0.00	0.00
60	0.00E+00	0.00E+00	0.00	0.00	0.00

S.eoftemberg at 68°C

1) 1st Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	1.01E+07	1.19E+07	7.00	7.08	7.04
0	5.90E+06	5.40E+06	6.77	6.73	6.75
5	3.60E+06	2.60E+06	6.56	6.41	6.49
10	2.31E+06	2.05E+06	6.36	6.31	6.34
15	1.22E+06	1.01E+06	6.09	6.00	6.05
20	8.30E+05	9.50E+05	5.92	5.98	5.95
25	6.60E+05	5.20E+05	5.82	5.72	5.77
30	4.70E+04	3.60E+04	4.67	4.56	4.61
40	1.90E+04	1.50E+04	4.28	4.18	4.23
50	9.80E+03	1.03E+04	3.99	4.01	4.00
60	5.00E+01	2.00E+01	1.70	1.30	1.50
70	3.00E+01	1.00E+01	1.48	1.00	1.24
80	2.00E+01	3.00E+01	1.30	1.48	1.39
90	0.00E+00	0.00E+00	0.00	0.00	0.00

2) 2nd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	1.41E+07	1.32E+07	7.15	7.12	7.13
0	7.00E+06	6.90E+06	6.85	6.84	6.84
5	2.60E+06	2.10E+06	6.41	6.32	6.37
10	1.90E+06	2.10E+06	6.28	6.32	6.30
15	1.80E+06	1.68E+06	6.26	6.23	6.24
20	9.30E+05	6.70E+05	5.97	5.83	5.90
25	1.80E+05	4.00E+05	5.26	5.60	5.43
30	7.80E+04	6.40E+04	4.89	4.81	4.85
40	6.40E+03	5.30E+03	3.81	3.72	3.77
50	9.80E+03	5.00E+03	3.99	3.70	3.85
60	3.20E+02	4.90E+02	2.51	2.69	2.60
70	6.00E+02	2.00E+02	2.78	2.30	2.54
80	7.00E+02	3.00E+02	2.85	2.48	2.66
90	0.00E+00	0.00E+00	0.00	0.00	0.00

3) 3rd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	1.07E+07	1.19E+07	7.03	7.08	7.05
0	8.20E+06	9.80E+06	6.91	6.99	6.95
5	7.40E+06	4.90E+06	6.87	6.69	6.78
10	5.50E+06	6.20E+06	6.74	6.79	6.77
15	2.39E+06	2.47E+06	6.38	6.39	6.39
20	2.34E+06	2.47E+06	6.37	6.39	6.38
25	1.93E+06	1.88E+06	6.29	6.27	6.28
30	9.00E+05	9.70E+05	5.95	5.99	5.97
40	5.00E+04	7.20E+04	4.70	4.86	4.78
50	MISSING	MISSING			0.00
60	8.00E+02	6.00E+02	2.90	2.78	2.84
70	1.80E+02	1.20E+02	2.26	2.08	2.17
80	5.00E+02	6.00E+02	2.70	2.78	2.74
90	6.00E+01	6.00E+01	1.78	1.78	1.78

LDH 68°C

1) 1st Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	6.77E+02	6.48E+02	2.83	2.81	2.82
5	6.51E+02	6.35E+02	2.81	2.80	2.81
10	7.35E+02	7.81E+02	2.87	2.89	2.88
15	6.35E+02	6.50E+02	2.80	2.81	2.81
20	6.42E+02	6.22E+02	2.81	2.79	2.80
25	4.93E+02	4.45E+02	2.69	2.65	2.67
30	4.12E+02	3.68E+02	2.62	2.57	2.59
40	2.62E+02	2.45E+02	2.42	2.39	2.40
50	2.32E+02	2.24E+02	2.36	2.35	2.36
60	2.14E+02	2.15E+02	2.33	2.33	2.33
70	2.31E+02	2.39E+02	2.36	2.38	2.37
80	1.35E+02	1.13E+02	2.13	2.05	2.09
90	1.54E+02	1.73E+02	2.19	2.24	2.21

2) 2nd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	7.33E+02	7.30E+02	2.87	2.86	2.86
5	6.67E+02	6.60E+02	2.82	2.82	2.82
10	6.62E+02	6.68E+02	2.82	2.82	2.82
15	6.14E+02	6.13E+02	2.79	2.79	2.79
20	8.86E+02	7.79E+02	2.95	2.89	2.92
25	7.57E+02	7.67E+02	2.88	2.88	2.88
30	7.46E+02	7.40E+02	2.87	2.87	2.87
40	5.02E+02	4.56E+02	2.70	2.66	2.68
50	4.21E+02	4.76E+02	2.62	2.68	2.65
60	1.94E+02	2.12E+02	2.29	2.33	2.31
70	2.49E+02	2.45E+02	2.40	2.39	2.39
80	2.39E+02	2.32E+02	2.38	2.37	2.37
90	7.30E+01	5.89E+01	1.86	1.77	1.82

3) 3rd Run

Time (sec)	1	2	Log 1	Log 2	Average
Raw	8.78E+02	8.95E+02	2.94	2.95	2.95
0	8.14E+02	8.53E+02	2.91	2.93	2.92
5	7.98E+02	8.03E+02	2.90	2.90	2.90
10	8.86E+02	8.44E+02	2.95	2.93	2.94
15	7.84E+02	7.82E+02	2.89	2.89	2.89
20	7.12E+02	8.09E+02	2.85	2.91	2.88
25	6.50E+02	6.40E+02	2.81	2.81	2.81
30	5.55E+02	5.69E+02	2.74	2.76	2.75
40	6.19E+02	5.54E+02	2.79	2.74	2.77
50	3.61E+02	3.12E+02	2.56	2.49	2.53
60	2.90E+02	2.83E+02	2.46	2.45	2.46
70	3.19E+02	2.56E+02	2.50	2.41	2.46
80	1.13E+02	1.23E+02	2.05	2.09	2.07
90	5.63E+01	5.72E+01	1.75	1.76	1.75