

**ISOLATION AND CHARACTERIZATION OF POTENTIAL
INDICATOR BACTERIA TO BE USED FOR VALIDATION OF
ESCHERICHIA COLI O157:H7 REDUCTION IN BEEF
SLAUGHTER PLANT CRITICAL CONTROL POINTS**

A Thesis

by

MARÍA BELEM MAGAÑA YÉPEZ

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

MASTER OF SCIENCE

August 2004

Major Subject: Food Science and Technology

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August 2004

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ABSTRACT

Isolation and Characterization of Potential Indicator Bacteria to Be Used for Validation of *Escherichia coli* O157:H7 Reduction in Beef Slaughter Plant Critical Control Points.

(August 2004)

María Belem Magaña Yépez, B.S., Universidad de Guanajuato

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Microbiological detection of foodborne pathogens is ineffective for monitoring critical control points (CCP) within a slaughter/processing Hazard Analysis and Critical Control Point (HACCP) system. Pathogens are usually absent from carcass surfaces and their uneven distribution makes it difficult to obtain a representative sample. However, microbiological testing can be applied within a HACCP plan to validate and verify the effectiveness of decontamination procedures designed to control hazards. With proper data collection, the reduction of an indicator group at a point in processing can indicate that a specific pathogen is being effectively controlled, especially when pathogen levels are too low to allow confirmation of process control, as they typically are in beef slaughter processing. Since *E. coli* O157:H7 has been shown to have some acid resistance, the ability of typical indicator organisms to accurately predict the reduction of this pathogen by carcass decontamination procedures has been a concern. Obtaining potential indicator bacteria from the same environmental reservoir as *E. coli* O157:H7 may provide non-pathogenic indicators with similar heat- and acid-resistance

characteristics suitable for use in processing plant environments for validation and verification of carcass decontamination treatments within HACCP plans.

Potential indicator bacteria were isolated from hides of cattle at slaughter facilities in Arizona, Georgia, and Texas and compared with isolates of *E. coli* O157:H7 from the same locations to determine similarity in acid- and heat-resistance characteristics. After evaluation at 2 heating temperatures (55 and 65°C) and 3 pH levels (3.0, 4.0, and 5.0), it was determined that several potential indicator bacteria were slightly more resistant than *E. coli* O157:H7 to heating and acid treatment. The greatest reduction in numbers for *E. coli* O157:H7 and indicator bacteria occurred at pH 3.0 and temperature of 65°C. Counts of bacteria grown at pH 4.0 and 5.0 were not significantly different.

Testing indicated that several of the isolates from cattle hides would make good process control indicators since the indicator bacteria were reduced by heating or acid conditions at similar or greater rates when compared to *E. coli* O157:H7, providing an increased level of security that pathogens have been reduced in processing.

DEDICATION

I would like to dedicate this work to my Mom, Yolanda Yépez López, for her unconditional love, for showing me that little sacrifices are just savings for a big payback in the future, to dream big and encouragement to follow them.

To my brothers William, Rodrigo, and Francisco, my sister Caridad for all their long distance support.

Last but not least, I would like to thank everyone I have met during this journey, for their understanding and confidence, through the good or bad moments, for those slaps on the wrist and support whenever I needed.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Gary Acuff for his trust in me in addition to his supervision and assistance during this work.

Thanks to Ms. Lisa Lucia (my American mom), for her invaluable assistance. I appreciate Dr. Judith Kells for helping to establish protocols to follow for this project.

Thank you to my lab mates, Lei, Elizabeth, and Kyle and to our wonderful student worker, Emily Ann, for their help through the long and tiring days of the project.

I want to extend appreciation to Dr. Lepori and Dr. Richter for their support and motivation, for providing me the vital first step of my long, but successful journey at Texas A&M University.

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INTRODUCTION

The first outbreak of *Escherichia coli* O157:H7 reported in United States occurred in 1982. Since then *E. coli* O157:H7 has been recognized as a major etiologic agent in hemorrhagic colitis and hemolytic uremic syndrome in humans. Foods such as ground beef, raw milk, apple cider and fermented hard salami have been involved in *E. coli* O157:H7 outbreaks. Recent studies have shown that cattle are considered the primary reservoir of *E. coli* O157:H7 which can be transferred to the animal carcass by fecal contamination during the slaughter process, as well as during the dehidating process if the hide makes contact with the exterior carcass surface. Detection of *E. coli* O157:H7 can be difficult because of high levels of other sorbitol non-fermenting bacteria, as well as the lack of sensitivity and specificity of the sampling methodology. New techniques such as immunomagnetic separation, latex agglutination, and m-PCR have increased sensitivity for the isolation and identification of *E. coli* O157:H7. Even though the origins and subsequent rates of *E. coli* O157:H7 carcass contamination have not been well established, the Hazard Analysis and Critical Control Point system has been used to decrease the risk of foodborne illness by intervening at various stages of the slaughter process that may pose a plausible risk of carcass contamination.

In the slaughter plant environment it is both impractical and inadvisable to directly measure the reduction of *E. coli* O157:H7 on carcasses. Therefore, a suitable indicator organism needs to be identified.

This thesis follows the style and format of International Journal of Food Microbiology.

The aim of this project was to isolate and characterize a library of potential indicator or surrogate bacteria to validate and verify microbial decontamination procedures used to improve the safety of fresh beef products. The objectives of this study were (1) isolation of *E. coli* O157:H7 and potential indicator bacteria from cattle hide, (2) determination of the level of resistance of isolated strains of *E. coli* O157:H7 to acid and heat, (3) comparison of the resistance of potential indicator bacteria to that of the *E. coli* O157:H7 strains most resistant to acid and heat.

LITERATURE REVIEW

***Escherichia coli* O157:H7 background**

The genus *Escherichia* is classified in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) as a Gram-negative, facultatively anaerobic rod. These bacteria are non-motile, or motile by peritrichous flagella, with an optimal growth temperature of 37°C. *E. coli* is oxidase negative, catalase positive, methyl red positive, Voges-Proskauer negative, citrate negative and also negative for hydrogen sulfide, urea hydrolysis, and lipase. *E. coli* is often subdivided serologically, or by the presence of virulence factors to identify and characterize epidemiologically pathogenic strains. Complete serotyping determination includes somatic (O), capsular (K), and flagellar (H) antigens.

E. coli is commonly found in human and animal intestinal tracts and is considered to be part of the normal microflora. However, some strains of *E. coli* have emerged as pathogens which are capable of causing severe disease and death in humans (Armstrong et al., 1996; Bell and Kyriakides 1998) (Table 1). Specifically, *E. coli* O157:H7 and O157: NM (non-motile) are recognized as major etiologic agents in hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) in humans (Schroeder et al., 2002).

Outbreaks

In 1982, *E. coli* O157:H7 was identified as a foodborne pathogen after being associated with the consumption of undercooked hamburger patties at a single fast-food restaurant. Illness was characterized by abdominal cramping, watery diarrhea followed

Table 1.

Serogroups and disease associations of six virulence types of *E. coli* (EC) (Bell and Kyriakides, 1998).

Virulence type	Serogroup examples	Disease association	Summary <i>E. coli</i> host interaction
Enteropathogenic (EPEC)	O18ab, O18ac, O26, O44, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, O158	Enteritis in infants; traveler's diarrhea	EPEC attach to intestinal mucosal cells causing cell structure alterations (attaching and effacing)
Enterotoxigenic (ETEC)	O6, O8, O15, O25, O27, O63, O78, O115, O148, O153, O159	Diarrhea, vomiting and fever; traveler's diarrhea	ETEC adhere to the small intestinal mucosa producing toxins that act on the mucosal cells
Vero cytotoxigenic (VTEC) (includes Enterohemorrhagic, EHEC)	O2, O4, O6, O8, O15, O18, O22, O23, O26, O55, O75, O91, O103, O104, O105, O111, O113, O114, O153, O163, O157, O168	<i>Shigella</i> -like dysentery (stools contain blood and mucus); hemolytic uremic syndrome	<i>E. coli</i> O157:H7 attach to and efface mucosal cells and produce toxin
Enteroinvasive (EIEC)	O28ab, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O164, O167, O173	<i>Shigella</i> -like dysentery	EIEC invade cells in the colon and spread laterally, cell to cell
Enteraggregative (EAggEC)	Not yet established	Persistent diarrhea in children	EAggEC bind in clumps (aggregates) to cells of the small intestine and produce toxins
Diffusely adherent (DAEC)	Not yet established	Childhood diarrhea	Fimbrial and non-fimbrial adhesions identified

by bloody diarrhea with little or no fever. The Centers for Disease Control and Prevention (CDC) have estimated that 73,000 cases of infection and 61 deaths due to *E. coli* O157:H7 occur each year in the United States, while there were 37,000 estimated cases of illness caused by non-O157 Shiga toxin-producing *E. coli* (CDC, 2000).

Foods such as apple cider (Steele and Murphy, 1982), ground beef, raw milk, (USDA: APHIS: VS 1997) and fermented hard salami have been implicated in *E. coli* O157:H7 outbreaks (Armstrong et al., 1996)

Pathogenesis

In the Foodborne Pathogenic Microorganisms and Natural Toxins Handbook, *E. coli* O157:H7 is defined as a specific *E. coli* serotype, expressing the O-antigen 157 and the H-antigen 7 (US-FDA 1992), which contains virulence attributes: ability to produce shiga-like toxins (SLT, also known as verotoxins or VT1 and VT2), adherence factor and enterohemolysis. The adherence factor enables the organism to attach to and colonize intestinal mucosal cells.

Large quantities of one or more related potent toxins that cause severe damage to the lining of the intestine have been associated with different human diseases. Some include mild diarrhea, hemorrhagic colitis, characterized by severe abdominal cramps, bloody stools, little or no fever, and evidence of erosion and hemorrhage of the colon, and also HUS (Schroeder et al., 2002). HUS can lead to hemolytic anemia (destruction of red blood cells), can affect the central nervous system, and can cause renal failure which is the most significant symptom. This disease occurs more frequently in children. Another disease related to these toxins is TTP, that is found in adults and has related

symptoms to HUS, with the exception of severe neurologic damage and other complications such as seizures, stroke or coma. Also, cause a decrease in the number of blood platelets, disorder that is characterized by purplish or brownish red discoloration, easily visible through the epidermis, caused by hemorrhage into the tissues (US-FDA 1992).

Virulence factors contributing to pathogenesis include the production of either or both of two phage encoded toxins and the formation of attaching and effacing lesions in the intestine of the host as was observed by McKee and O'Brien (1995). Their observations also included the reduction in the adherence capabilities of *eaeA* mutants.

Transmission

Ruminant animals such as cattle, goats, deer and sheep are considered carriers of *E. coli* O157:H7 (Doyle, 1991). Reports have implicated cattle as the primary reservoir of *E. coli* O157:H7, but initial prevalence surveys conducted on fed cattle estimated the overall fecal prevalence of *E. coli* O157:H7 to be very low (Chapman et al., 1992; Hancock et al., 1997b). However, more recent studies using improved enrichment and isolation procedures have indicated that the overall prevalence of *E. coli* O157:H7 in cattle may be significantly higher than originally estimated (Heuvelink et al., 1998). It is also known that animals carrying *E. coli* O157:H7 are asymptomatic and a visual examination will not make a distinction between carriers and non carriers. Recent studies have shown that the incidence of this pathogen on the hides of cattle presented for slaughter can be as high as 11% (Elder et al., 2000) or 22% (Reid et al., 2002a).

Contamination can be spread between animals during transportation, either directly via body contact or indirectly via contact with contaminated floors/surfaces (particularly the brisket area) (Reid et al., 2002b). Consequently, *E. coli* O157:H7 can be transferred to the animal carcass by fecal contamination during the slaughter process (Sheridan, 1998), and also during the dehiding process if the hide makes contact with the exterior carcass surface (Wells et al., 1983; Gill and McGinnis, 1999).

Isolation procedures

Selective and differential media have been developed to facilitate the isolation of *E. coli* O157:H7. The addition of antimicrobial supplements such as potassium tellurite, cefixime and novobiocin inhibit background microflora which are likely to be present in higher numbers than *E. coli* O157:H7.

E. coli O157:H7 can be distinguished from non-O157 by two biochemical characteristics in which strains are not capable of forming acid from sorbitol or producing β -D-glucuronidase (Aleksic et al., 1992). Sorbitol MacConkey (SMAC) selectivity is improved with the addition of cefixime-tellurite (CT-SMAC) (Zadik et al., 1993). The sorbitol in this media helps to identify the suspected colonies of O157:H7, which are colorless on this medium (Manafi and Kremsmaier, 2001).

Rainbow[®] agar has both selective properties and chromogenic substances specific for two enzymes generally related to *E. coli*. The substrate for β -galactosidase is blue-black and that for β -glucuronidase is red, making it particularly useful for isolating pathogenic strains such as O157:H7, *E. coli* O157:H7 are typically glucuronidase-

negative, and form distinctive charcoal gray or steel black colonies (Bettelheim, 1998). Rainbow[®] agar's selectivity is enhanced when supplemented with novobiocin (Stein and Bochner, 1998). CHROMagar[®] supplemented with potassium tellurite is a chromagenic selective medium that is rapid and reliable in the detection of the enterohemorrhagic *E. coli* O157. The colonies are easily distinguishable due to the purple coloring because of the production of β -galactosidase. Most other bacterial species are inhibited, giving blue or colorless colonies (Reissbrodt, 1998) because of the production of other products. A latex confirmation test for O157 is suggested for suspect colonies.

Screening tests

Screening commercial tests such as ELISA (enzyme-linked immunosorbent assay) detect the *E. coli* O157 antigen, but prior enrichment is required (Cray et al., 1998). Immunomagnetic separation (IMS) has been suggested as a successful method of reducing total analysis time and improving sensitivity for detection of pathogens (Verzony-Rozand, 1997). This technique is based on the interaction between antibodies immobilized onto paramagnetic particles and antigens in the samples through H-bonds and other intermolecular interactions. After the enrichment of *E. coli* O157:H7, IMS is recommended followed by plating onto selective agars.

The latex agglutination procedure is simple to perform, very efficient, and consistent in identifying *E. coli* O157:H7. This is a rapid slide test in which latex particles coated with a specific antibody react with the corresponding *E. coli* antigen such as O157 or H7 (March and Ratman, 1989).

The presence of shiga toxin is confirmed using a commercial toxin assay such as Premier EHEC Kit[®] (Meridian Diagnostics Inc., Cincinnati, OH) which is a rapid test for detection of all Shiga toxins produced by Shiga toxin-producing *E. coli* (STEC).

Confirmation of carriage of toxin genes can be performed using the polymerase chain reaction (PCR). Genes commonly detected in PCR for *E. coli* O157:H7 are *eaeA* (gene that encodes intimin), *ehxA* (gene encoding the hemolysin) (Nataro and Koper, 1998), *sxt1*, *sxt2* (shiga toxin 1 and 2; respectively) and *rfbO157* (*E. coli* antigen) (Osek, 2002). Intimin is a bacterial external membrane protein whose purpose is to mediate close contact between the bacteria and the target host cell (Frankel et al., 1998).

Prevention strategies adopted by the food industry

Even though the origins and subsequent rate of *E. coli* O157:H7 carcass contamination have not been well established (Wells et al., 1983), the Hazard Analysis and Critical Control Point (HACCP) system has been used to decrease the risk of foodborne illness by intervening at different stages of the slaughter process that pose a plausible risk of carcass contamination. The principal HACCP objective for raw foods focuses on reducing and preventing microbial growth.

Many decontamination methods have been investigated for their capability to reduce pathogens, and current microbial interventions primarily include organic acids (Castillo et al., 2001), non-acid compounds such as trisodium phosphate (TSP) (Bender and Brotsky, 1992) and thermal treatments such as hot water (Smith, 1992) and steam pasteurization (Wilson and Leising, 1994). Previous studies indicate that lactic or acetic

acid sprays, applied at 55°C, can reduce levels of *Salmonella* and *E. coli* O157:H7 (Hardin et al., 1995 and Castillo et al., 1998, 2001). Decontamination methods may decrease the number of the pathogenic bacteria, but recontamination could occur at any point during fabrication and distribution for retail.

Since acid pH is an important factor influencing the ability of pathogens to cause disease, some organic acids, such as acetic, citric, lactic, fumaric, propionic, and malic acid, are added to foods to prevent or delay the growth of pathogenic or spoilage bacteria (Dziezak, 1986; Everis, 2001). However, some pathogens, such as *E. coli* O157:H7, are reported to have high acid tolerance and could potentially survive for longer periods in acidic foods, enhancing their acid resistance compared to other pathogens and even compared to other *E. coli* strains (Goodson and Rowbury, 1989; Hill et al., 1995; Duffy et al., 2000).

Many factors can influence the ability of *E. coli* O157:H7 to survive under acidic conditions, including acid pre-adaptation, incubation temperature, and phase of bacterial growth. Of concern to the food industry is that pathogens may become stress-acid adapted when exposed to sublethal levels of a stress such as acid or heat during decontamination procedures and become more resistant to subsequent stresses. In addition, a cross-protection phenomenon is often observed, where exposure to one stress leads to an increased resistance to subsequent exposure to different stress. For example Wang and Doyle (1998) found that sublethal heat treatment of *E. coli* O157:H7 cells substantially increased their tolerance to acid. When microbial interventions are used, it is essential that the slaughter plant verify that the critical limits they have set (such as pH

of the acid or temperature of the water) are effective in actually killing any *E. coli* O157:H7 cells present on the carcass. Otherwise, exposure to the decontamination method at sublethal levels may simply lead to the development of stress-acid adapted *E. coli* O157:H7 cells, and create a potential food safety risk.

Indicator organisms

In the slaughter plant environment it is both impractical and inadvisable to attempt to directly measure the reduction of *E. coli* O157:H7 on carcasses. Therefore, a suitable indicator organism needs to be identified. Jay (2000) suggested that the ideal indicator used to assess food safety should meet the following criteria: 1) be easily and rapidly distinguishable from other members of the food flora, 2) have a history of constant association with the pathogen whose presence it is to indicate, 3) always be present when the pathogen of concern is present, 4) be an organism whose number ideally should correlate with those of the pathogens, 5) have a die-off rate that at least parallels that of the pathogen and ideally persists slightly longer than the pathogen of concern and 6) be absent from foods that are free of the pathogen except perhaps at certain minimum numbers. The purpose of this study was to identify an indicator microorganism that existed in the same environment and had the same acid and heat resistance properties as *E. coli* O157:H7, with the ultimate aim to use this organism to validate and/or verify the effectiveness of decontamination procedures used in individual slaughter plants.

MATERIALS AND METHODS

Sample collection

One-hundred hide samples were collected from small, medium and large slaughter facilities in Texas (Amarillo, Bryan, Corpus Christi, San Angelo, San Antonio and Temple), Georgia (Augusta) and Arizona (Phoenix) by personnel from Texas A&M University (College Station, TX).

Sponge sampling technique

SpongeSicles (International BioProducts, Redmond, WA) were rehydrated with 25 ml Butterfield's Buffer (International BioProducts). Excess liquid was expelled from the sponge and it was aseptically removed from the bag. The hide surface area was swabbed near the ventral brisket (Reid et al., 2002a) area by swabbing 10 times horizontally and 10 times vertically within an estimated 100-cm² area. Hides were sampled immediately after stunning and before dehidng.

Sample analysis

Immediately after sampling, the sponge end of the SpongeSicle was returned to the sterile Whirl-Pak[®] bag (Nasco 710 ml, International BioProducts), the plastic handle was snapped off and discarded, and the Whirl-Pak[®] bag was sealed. Samples were transported to the Texas A&M University Food Microbiology Laboratory (College Station, TX) under refrigerated conditions by placing them in an ice chest (47.6 X 30.1 X 31.1 cm ThermoSafe[®] Multipurpose Insulated Bio-Polyfoam Shipper; Model 494,

Polyfoam Packers Corp., Wheeling, IL) containing UTEK[®] +30°F frozen refrigerant packs (360 ml or 1500 ml Polyfoam Packers Corp.). Samples were analyzed 24 h post collection. Each sample was double bagged by placing the first Whirl-Pak[®] bag containing the sponge inside a second sterile Whirl-Pak[®] bag (International BioProducts), and stomached (Stomacher Lab-Blender 400, Tekmar[®] Company, Cincinnati, OH) for 1 min. The contents were simultaneously analyzed for *E. coli* O157:H7 and potential indicators (Fig. 1).

Indicators

Isolation of indicator organisms was determined by plating 0.1 ml of the sample homogenate and then 0.1 ml of appropriate 10-fold dilutions of the same prepared and dried Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI) plates and also Violet Red Bile Agar (VRBA, Difco) plates. Samples were spread over the surface of the plates using a sterile bent glass rod. The TSA and VRBA plates were incubated at 37°C for 24 h.

Initial acid resistance of the potential indicators was evaluated using a replica-plating technique to screen for growth on TSA plates adjusted to pH 4.5 and 5.0 (TSA-pH 4.5 and TSA-pH 5.0) with 88% L-lactic acid (Purac America Inc., Wood Dale, IL). TSA and VRBA plates exhibiting good growth as well-separated colonies were chosen as master plates. A mark was placed on the master plate with a sharpie (TSA or VRBA) and on the TSA-pH 4.5 and the TSA-pH 5.0 test plates for orientation. Using a VWR replica-plating tool (VWR International, Suwanee, GA) covered with a sterilized

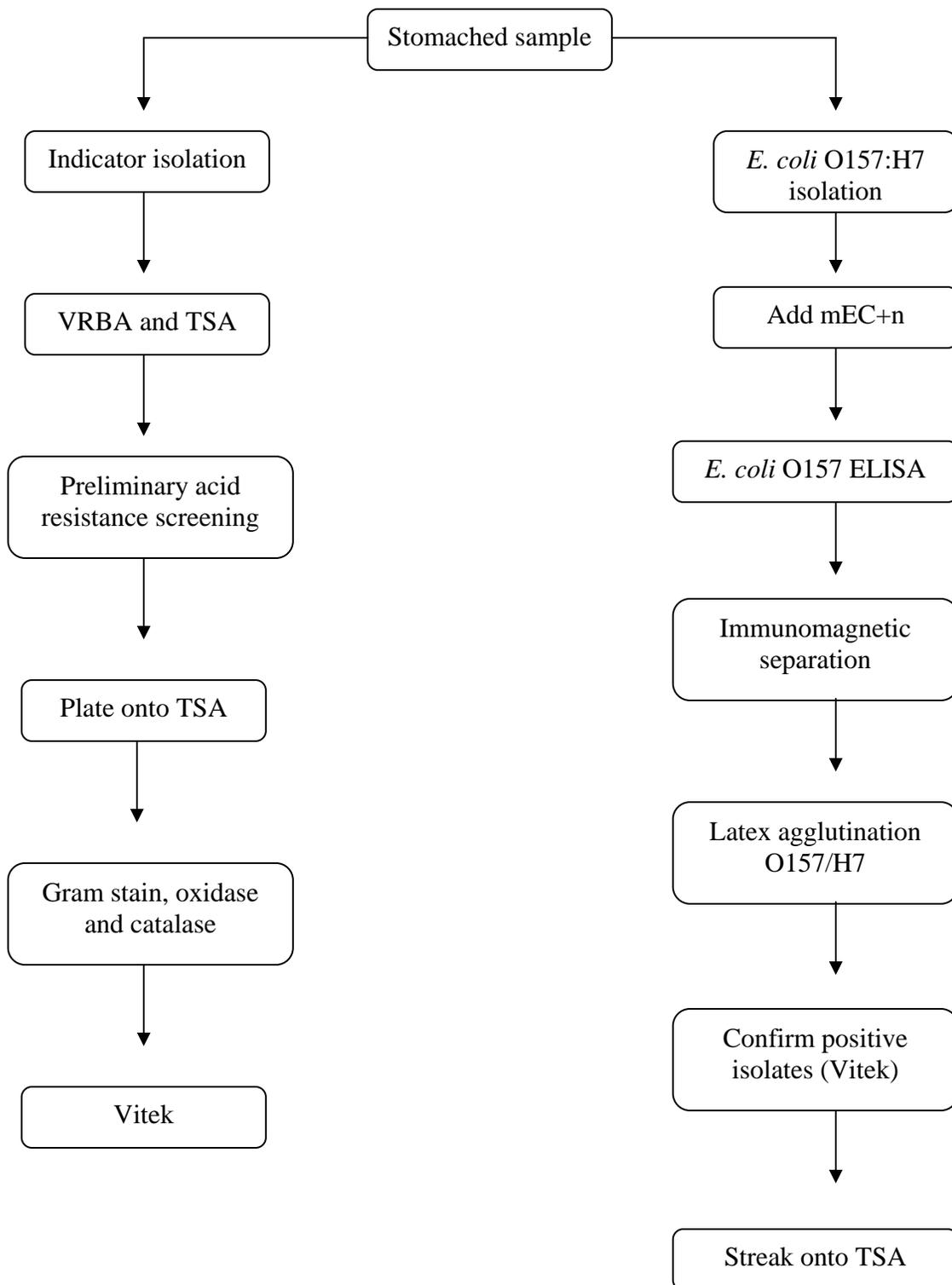


Fig. 1. Analysis for *E. coli* O157:H7 and potential indicators

velveteen square (VWR), an imprint was made of the master plate on the TSA-pH 4.5 and TSA-pH 5 test plates. A locking ring secured the velveteen square to the cylinder just prior to replica plating. The selected petri dish containing the colonies was inverted and lowered onto the velveteen surface, then removed. Replication was made by applying a fresh nutrient agar surface to the velveteen square with the culture. The replica-plating tool was disinfected with 70% alcohol between uses. The test plates and master plates were incubated at 37°C for 24 h.

Potential indicator bacteria demonstrating acid resistance by growth on TSA-pH 4.5 and TSA-pH 5.0 were carried forward for further characterization. The corresponding colony was selected from the master plate, streaked to TSA and incubated at 37°C for 24 h to check for purity. If the culture was pure, Gram stain, oxidase test, and catalase test on single, well-isolated colonies were performed. The isolates were streaked onto blood agar [TSA base plus 5% defibrinated sheep blood (Cleveland Scientific, Bath, OH)] and incubated at 37°C for 24 h.

Selected colonies from blood agar plates were suspended into 2.0 ml of 0.45% sterile saline solution (Baxter Healthcare, Deerfield, IL) in a 12 X 75-mm clear, sterile, disposable tube (International BioProducts) and vortexed (Vortex Genie™; Scientific Industries Inc., Bohemia, NY) for homogenization. The turbidity of the inoculum was measured using a colorimeter (Model DR 100; HACH Company, Loveland, CO) to the equivalent of a No. 1 McFarland turbidity standard. The identity of the potential indicators was verified by biochemical characterization using a Vitek Gram-negative or Gram-positive identification card (GNI⁺ or GPI card; BioMérieux Vitek Inc.,

Hazelwood, MO). Colonies were streaked to TSA and incubated at 37°C for 24 h before storage on cryogenic beads (Protect™, Key Scientific Products, Round Rock, TX) at -80°C.

E. coli O157:H7

The remaining sample was subjected to enrichment and IMS separation in order to examine for and confirm the presence of *E. coli* O157:H7.

EC broth (International BioProducts), containing 20 mg/l novobiocin (Sigma Chemical Co., St. Louis, MO) (mEC+), was added to each whirl-pack bag to increase the volume to 250 ml, and then stomached for 2 min. Positive, negative, and inoculated media controls were also included as described in the USDA/FSIS protocol (Cray et al., 1998). Samples were incubated at 37°C for 24 h. Enriched samples were screened for *E. coli* O157 using the Tecra® *E. coli* O157 VIA (Tecra International Pty Ltd, Chatswood, NSW, Australia) following manufacturer's instructions.

Positive samples were subjected to IMS separation using Dynabeads® anti-*E. coli* O157 (Dynal Inc., Lake Success, NY) according to the manufacturer's instructions with the exception that 30 µl of each sample was plated onto 3 selective agars: Rainbow® agar O157 (Biolog Inc., Hayward, CA) containing 10 mg/l novobiocin (Sigma) and 0.8 mg/l potassium tellurite (Sigma) (Cray et al., 1998; Manafi and Kremsmaier, 2001), CHROMagar® O157 (Dynal Inc.) containing 2.5mg/l potassium tellurite (Sigma), and Sorbitol-MacConkey agar (CT-SMAC, Difco) supplemented with 2.5 mg/l potassium tellurite and 0.05 mg/l cefixime (Dynal Inc).

When possible, 5 suspect colonies were selected from each selective agar and latex agglutination was performed for O157 utilizing the RIM[®] *E. coli* O157:H7 test (Remel, Lenexa, KS) according to the manufacturer's instructions. Latex positive isolates were streaked to blood agar and incubated at 37°C for 24 h. Using growth from the blood agar plate, *E. coli* O157:H7 isolates were confirmed according to the USDA/FSIS method (Cray et al., 1998). Biochemical confirmation using GNI⁺ (Vitek) was performed as well as conventional tube media tests (Triple Sugar Iron Agar (TSIA), cellobiose and motility test medium) in order to differentiate between *E. coli* O157 and similar competitive organisms. Shiga toxin/toxin genes confirmation was performed by shiga toxin assay using a Meridian Premier EHEC Kit[®] (Meridian Diagnostics Inc.) according to the manufacturer's instructions, and multiplex PCR (mPCR) following the method described by Osek (2002).

If the isolate was confirmed as *E. coli* O157:H7, or *E. coli* O157:NM (or H Indeterminate) and the Shiga toxin(s) and/or one or more toxin genes were present, the sample was treated as positive for *E. coli* O157:H7 (Cray et al., 1998). Isolates (from TSA plates) were stored on cryogenic beads (Protect[™]) at -80°C.

Characterization

The resistance of acid-acid adapted (A) and non-acid adapted (NA) *E. coli* O157:H7 cells to pH-adjusted phosphate buffered saline was determined using a method based on Buchanan and Edelson (1996) and Berry and Cutter (2000). Heat resistance testing was acid adapted from Jackson et al. (1995). The experiments were performed

using 6 randomly selected *E. coli* O157:H7 hide isolates, and the number of parameters measured was adjusted according to the results obtained. The level of resistance of potential indicators was compared to that of the most heat-resistant and acid-resistant *E. coli* O157:H7 isolated from cattle hide in this study.

In order to determine when the cells reached stationary phase, a growth curve was constructed by placing a stored cryogenic bead (Protect™) at -80°C into 10 ml Tryptic Soy Broth (TSB) and incubating overnight at 37°C. After incubation, 0.1-ml aliquots were used to inoculate tubes containing 10 ml TSB and TSB enriched with 1% glucose (TSB+G, Difco). Over a 24-h period, one tube was removed for sampling every hour. A dilution series was prepared and TSA plates were inoculated for enumeration. Colonies were counted after incubation at 37°C for 24 h. Isolates grown in TSB were considered as NA cells, and those isolates grown in TSB+G considered A cells.

Acid resistance of the A or NA *E. coli* O157:H7 cells were evaluated in Phosphate Buffer Saline solution (PBS), (US-FDA, 2001), adjusted to pH 2.0, 3.0, 4.0 and 5.0 and pre-tempered to room temperature. The stationary phase A or NA cultures were washed once in 10 ml PBS, and 0.1-ml aliquots used to inoculate 8 tubes containing pH-adjusted PBS. Over a 7-h period, one tube was removed for sampling every hour. A dilution series was prepared and plates were inoculated on TSA and TSA + 4% NaCl, as described earlier. Colonies was counted after incubation at 37°C for 24 h in order to determine the number of surviving, injured and killed cells after stress.

To measure heat resistance, NA stationary phase cultures were prepared in TSB and re-suspended in 10 ml PBS as before. Volumes of 0.1 ml were transferred to

watertight vials (Micro Sample Vial, Screw cap, 19 X 65 mm, American Scientific Products, McGaw Park, IL) containing 3.9 ml PBS pre-tempered to 55 or 65°C. Vials were placed in a water bath (Magni Whirl[®] Constant Temperature Bath; Blue M.; Blue Island, IL) at 55 or 65°C, and 1 vial was removed every 2 min for up to 20 min and chilled immediately in iced water for 30 s. Dilutions were prepared and plate counts were conducted as described before.

During the heat resistance testing the temperatures in the water bath and vials were recorded using thermocouples (Type K; Pico Technology Limited, St. Neots, Cambridgeshire, UK) connected to the thermocouple data logger (Pico Technology Limited). Temperature data for the data logger was then downloaded to a computer and analyzed with the supplied software (Pico Technology Limited) which provided a macro to collect data directly into an Excel Spreadsheet (Microsoft Excel; Microsoft, Bellevue, WA).

Statistical analyses

Microbiological plate counts were converted to \log_{10} CFU/ml before analysis. Data were analyzed using the general linear model (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, N.C.). A $P < 0.05$ value was used to determine whether there were significant differences in the analysis of treatment effects.

RESULTS AND DISCUSSION

A total of 100 hide samples were collected from the ventral brisket area of cattle immediately prior to slaughter at commercial slaughter facilities located in Texas (San Angelo, San Antonio, Temple, Corpus Christi, Amarillo, and Bryan), Augusta, GA and Phoenix, AZ.

Hides are known to be a primary source of *E. coli* O157 contamination of beef and the bacteria can be transferred onto the carcass surface from the hide during slaughter and dressing. The high prevalence of *E. coli* O157 on the brisket area may be attributed to the animals lying down on contaminated ground either on the farm, during transport, or by contact with the floor inside the stunning box (Elder et al., 2000, Reid et al., 2002a). The initial cut during dehiding passes centrally through the brisket posing greatest risk of carcass surface contamination (Reid et al., 2002a). Previous studies reported that a visibly clean hide may not necessarily be pathogen free (Newton et al., 1978; Ridell and Korkeala, 1993; McEvoy et al., 2000). Results of this study supports those published findings, in which most of the cattle sampled were observed to be clean, but hides were found contaminated with *E. coli* O157. Smith et al. (2001) reported the prevalence of cattle shedding *E. coli* O157:H7 varied greatly between pens in each feedlot. Reid et al. (2002b) demonstrated varied levels of the bacteria may be due to differences in fecal shedding of individual animals, or differences in survival rates of bacteria either on the hide and/or animal-related environments. A number of studies have revealed significant variation in the number of animals shedding *E. coli* O157 in feces (1-11%) which would directly translate to variation on hides (Hancock et al.,

1997a; Fedorka-Cray et al., 1998).

E. coli O157:H7

Screening tests

Fifty percent of the hide samples screened for *E. coli* O157 using ELISA were positive. These positive samples were further characterized for *E. coli* O157 by immunomagnetic separation (IMS). 10% of the samples tested by latex agglutination were positive for *E. coli* O157:H7 and 9% for *E. coli* O157, having an overall occurrence of 19% *E. coli* O157. This percentage can be compared with those previously reported by Reid et al. (2002b) who found 22.2% of *E. coli* O157 and Elder et al. (2000) who found 38% of lots tested to have at least one hide positive for *E. coli* O157, with 11% overall of *E. coli* O157 prevalence on hides of cattle presented for slaughtering.

Biochemical and presences of toxin confirmation

After confirmation by VITEK, 16% of isolates were found to be *E. coli* O157:H7. The presences of toxins were confirmed for all isolates by the toxin assay (EHEC). In addition, mPRC showed the presence of genes commonly detected for *E. coli* O157:H7 as *eaeA* (intimin attachment gene), *stx1*, *stx2* and *rfbO157* (gene for the O157 antigen) (Fig. 2).

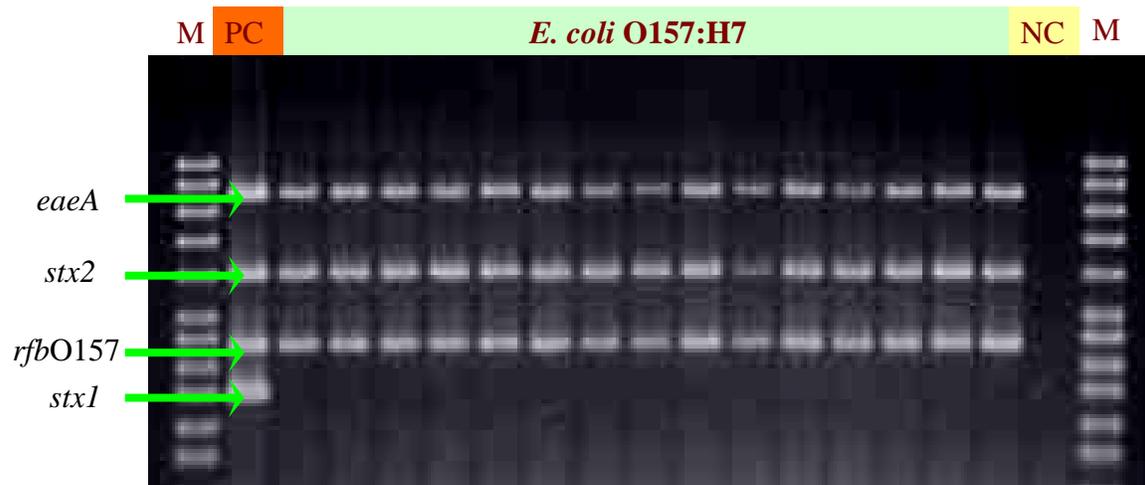


Fig. 2. mPCR pattern of genes commonly detected for *E. coli* O157:H7
eaeA (840 bp), *stx2* (584 bp), *rfbO157* (420 bp) and *stx1* (348 bp)

Characterization of *E. coli* O157:H7:

Acid resistance

Buchanan and Edelson (1996) used the terms “acid tolerance response” and “acid habituation” to describe pH dependent tolerance, and acid resistance to describe pH independent, stationary growth phase dependent tolerance. In absence of glucose, cell growth declined between 0.3 and 3.8 logs due to acid-resistant differences among bacterial strains. However, induced acid-tolerance response in *E. coli* O157:H7 was achieved by adding glucose to TSB with a final pH of 4.8. Everis (2001) found that many factors influence the ability of *E. coli* O157:H7 to survive under acidic conditions. Such factors include whether the cells have been pre-acid adapted or acid shocked, the temperature at which the cells are incubated, the phase of the bacterial growth cycle, the strain of *E. coli* O157:H7 studied, and the acidulant type and concentration.

Garren et al. (1997) described the acid tolerance response as a two-stage process where there is an initial exposure to a mild pH (pH 5.0-6.0), known as acid adaptation, and then acid shock at pH 4.0. This acid shock response can produce enhanced acid resistance after a switch from low acid to high acid conditions.

The resistance of bacteria such as *E. coli* to environmental stresses is recognized as an increasingly important area of microbiology. In particular, the observation that cells in the stationary phase of growth are more resistant to many environmental stresses than their counterparts in the exponential phase has received much attention (Dodd and Aldsworth, 2002). This particular behavior reflects differential sensitivity of *E. coli* which might be considered to establish specific heat treatments such in water for

decontamination of beef carcasses, and acidified solution-based treatments (Hengge-Aronis et al., 1991; McCann et al., 1991; Brackett, et al., 1994; and Lee et al., 1995). Growth curves at 12 and 18 h were carried out on 3 randomly selected O157:H7 isolates (C1, C2, and C3) to determine the stationary phase for each isolate. This evaluation used acid adapted (A) and non-acid adapted (NA) isolates. For these isolates it was determined that the stationary phase was reached after 7 h incubation, independent of the initial inocula (Figs. 3 and 4). At the end of the growth curve the final pH was 4.8, this pH was the same found by Buchanan and Edelson (1996). Although the three isolates did not differ significantly when grown under acid adapted conditions, under non-acid adapted conditions C1 was significantly different from C2 and C3 (Tables 2 and 3). Arnold and Kaspar (1995) also illustrated that there was no difference in acid adapted and non-acid adapted cells of *E. coli* O157:H7 with respect to survival of an acid challenge, and showed that although *E. coli* O157:H7 has strain-to-strain variation in acid resistance it is more resistant than generic strains. Studies by Ryu and Beuchat (1998) suggested that *E. coli* O157:H7 could be acid adapted to acid conditions and exhibited enhanced survival as well as survived acid shock when introduced into acid environments with no previous adaptation. Deng et al. (1999) compared the growth of strains of *E. coli* O157:H7 under acid adapted and non-acid adapted conditions and showed no difference in pattern of growth when the same acidulant and same pH were compared. Cells that multiply during fermentation of foods such as salami or yogurt may undergo acid adaptation but not acid shock (cells have not grown in an acid environment) due to gradually decrease in their pH (Ryu and Beuchat 1999). The level

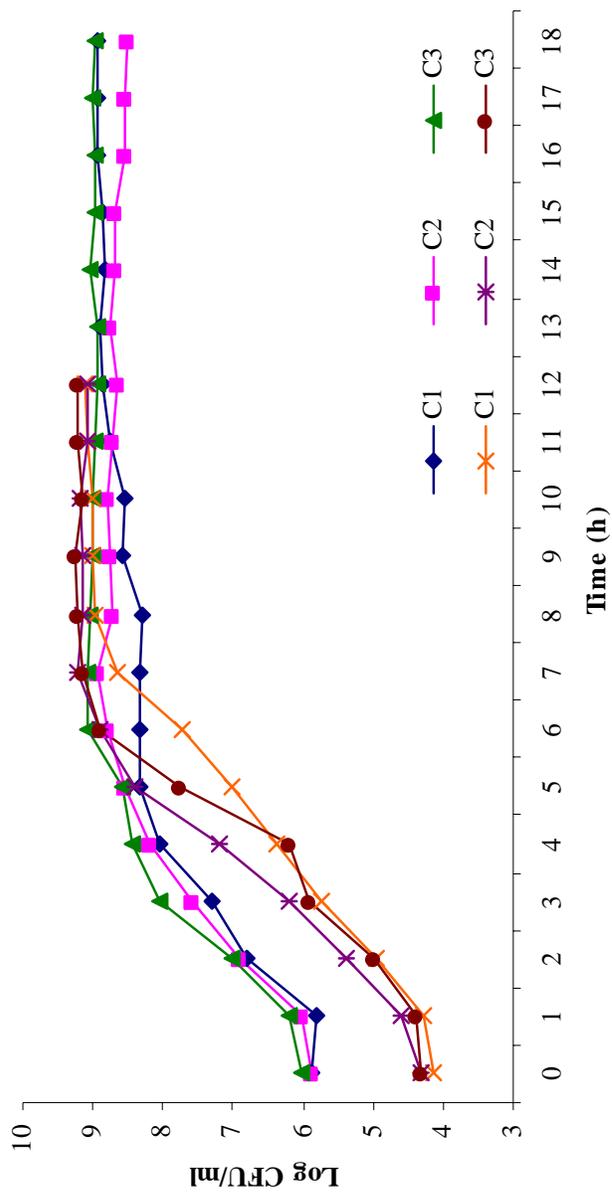


Fig. 3. Viable counts for *Escherichia coli* O157:H7 harvested every hour from TBS + 1% glucose (acid adapted) incubated at 37°C during 12 h and 18 h to determine stationary phase.

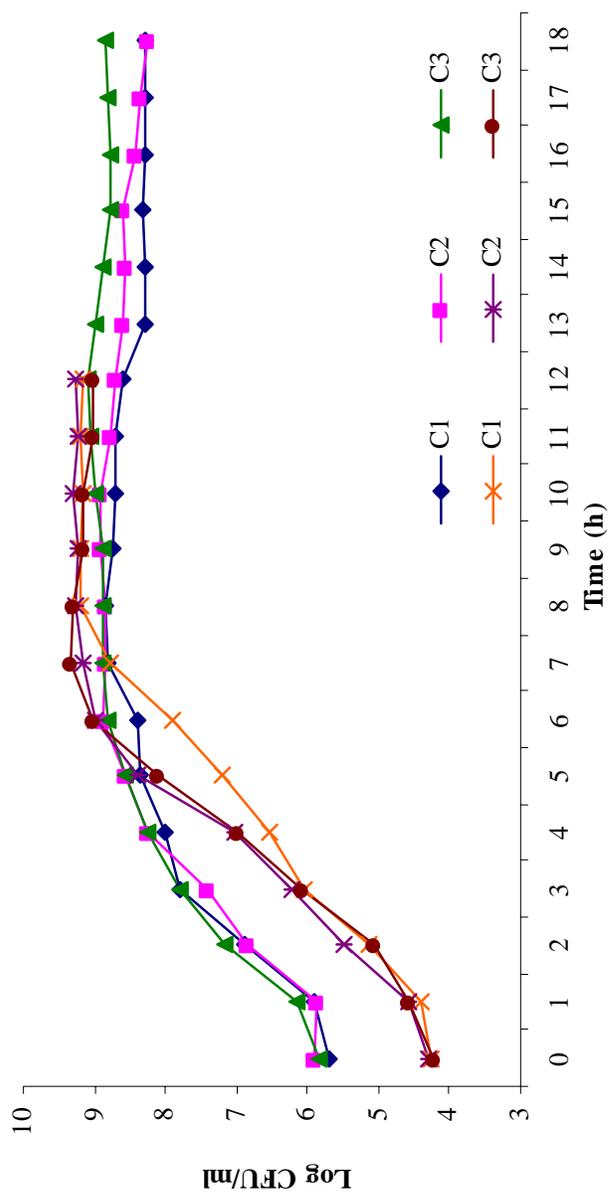


Fig. 4. Viable counts for *Escherichia coli* O157:H7 harvested every hour from TBS without glucose (non-acid adapted) incubated at 37°C during 12 h and 18 h to determine stationary phase.

Table 2.

Final population of *E. coli* O157:H7 under acid adapted conditions incubated at 37°C during 12 h and 18 h to determine growth curve.

Isolate	Log CFU/ml
C1	8.8 ^a
C2	8.9 ^a
C3	8.9 ^a

^aValues for each isolated with the same letter (a or b) are not significantly different (P < 0.05).

Table 3.

Final population of *E. coli* O157:H7 under non-acid adapted conditions incubated at 37°C during 12 h and 18 h to determine growth curve.

Isolate	Log CFU/ml
C1	8.6 ^a
C2	8.8 ^b
C3	9.2 ^b

^aValues for each isolate with the same letter (a or b) are not significantly different (P < 0.05).

of acid resistance of *E. coli* O157:H7 has been suggested to correlate with the infectious dose required for this organism to cause disease in humans (Gordon and Small, 1993). Aldsworth et al. (1998) found an enhanced resistance of exponential phase cells of *E. coli*. This enhancement of resistance in exponential phase cells was of the same order of magnitude as that shown by stationary phase cultures of the same organism. Therefore, for this study, the resistance of acid-acid adapted and non-acid adapted for *E. coli* O157:H7 cells were performed at their stationary phase after 18 h. This time was chosen to assure that cells were already in the stationary phase for the determination of the level of resistance of isolated strains of *E. coli* O157:H7 to acid.

Cheng et al. (2003) found that acid-acid adapted cells showed a higher survival percentage than the non-acid adapted cells and that the most acid adaptation response was observed at pH 3.0, followed by pH 4.0 and 5.0. Tetteh and Beuchat (2003) found that a lower number of colonies formed on TSAN indicated the presence of injured cells that could not be revived and cells inoculated into acidified TSB at pH 3.5 were not able to grow on TSAN. Similar results were found in this study. Cells acidified in TSB at pH 3.0 were not detected to grow on TSAN after 2-h incubation. Following the same tendency, higher populations of all three isolates (C1, C2, and C3) were recovered only on TSA and not TSAN at pH 3.0. The minimum detection limit was determined to be 1.7 log/ml, indicating <1 CFU/ml. Brackett et al. (1994) used lactic acid to acidify TSB which was the most effective, and the only acid to have a considerable effect on decreasing initial populations on *E. coli* O157.

No significant differences in colonies formed were observed at pH 4.0 and 5.0 on TSA or TSAN for both acid adapted and non-acid adapted conditions. This result indicated that no injury was observed as compared to the difference observed at pH 3.0. These results can be compared with those found by Tetteh and Beuchat (2003) who did not find significant differences in numbers of colonies formed on TSA and TSAN for *Shigella. flexneri* at a pH between 5.0 and 7.3.

For pH 3.0 the bacterial counts decrease approximately 6 log after 2 h for both acid adapted and non-acid adapted conditions (Figs. 5 to 8). Isolates C1 and C3 were significantly different under acid adapted but not under non-acid adapted conditions when plated on TSA. In general, strains of *E. coli* O157:H7 were most sensitive at pH 3.0, which value was selected for further comparison of the resistance of potential indicator bacteria.

The initial bacterial counts for pH 4.0 and pH 5.0 under acid adapted and non-acid adapted conditions were approximately 8.9 log. After 2 h incubation, the counts were approximately 8.8 log indicating no significant difference at pH 4.0 and 5.0 (Figs. 9 to 16) for either acid adapted or non-acid adapted conditions.

Heat resistance

Successful elimination of *E. coli* O157:H7 from food systems during processing has been achieved by applying heat under satisfactory conditions of time and temperature (Czechowicz, et al., 1996). Li Wan Po et al. (2002) demonstrated that an increase in

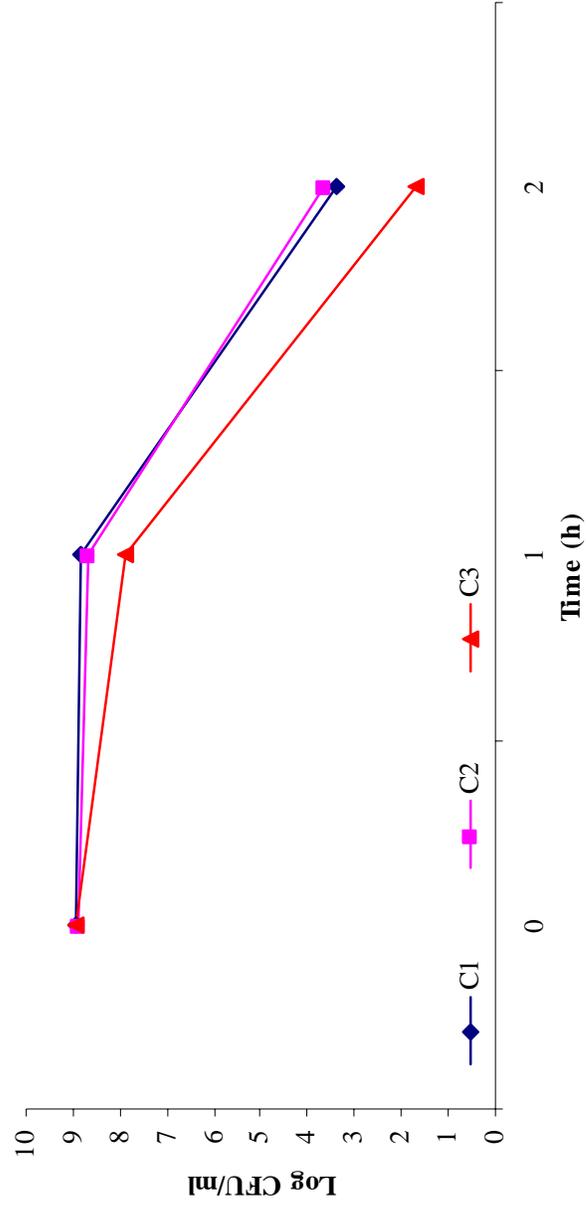


Fig. 5. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSA.

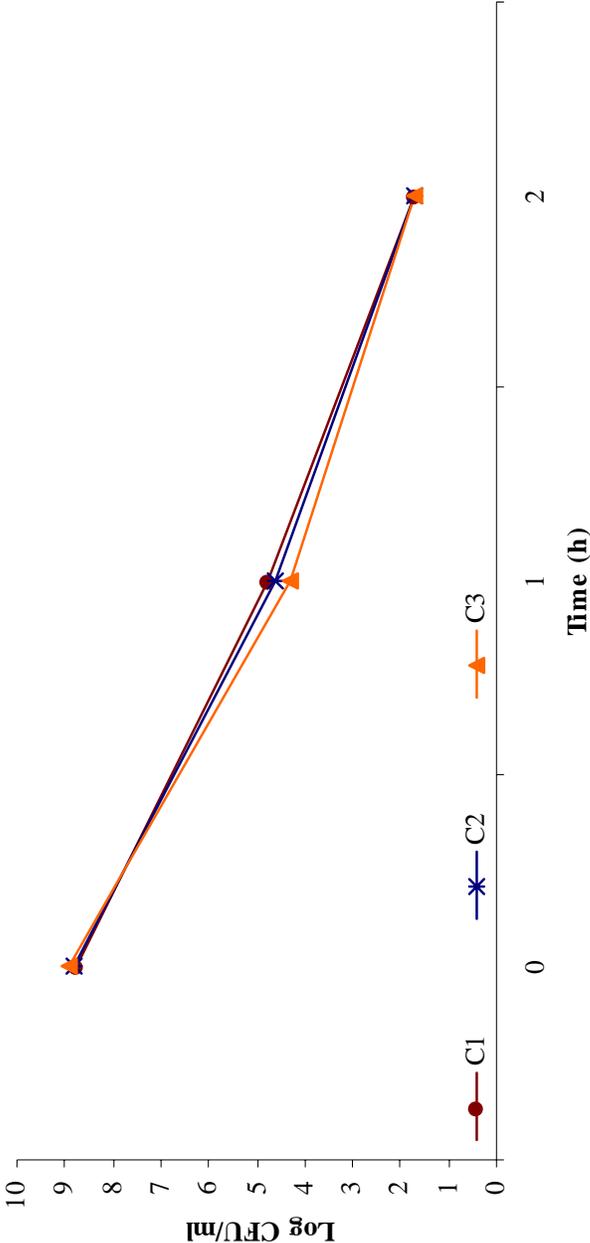


Fig. 6. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSAN.

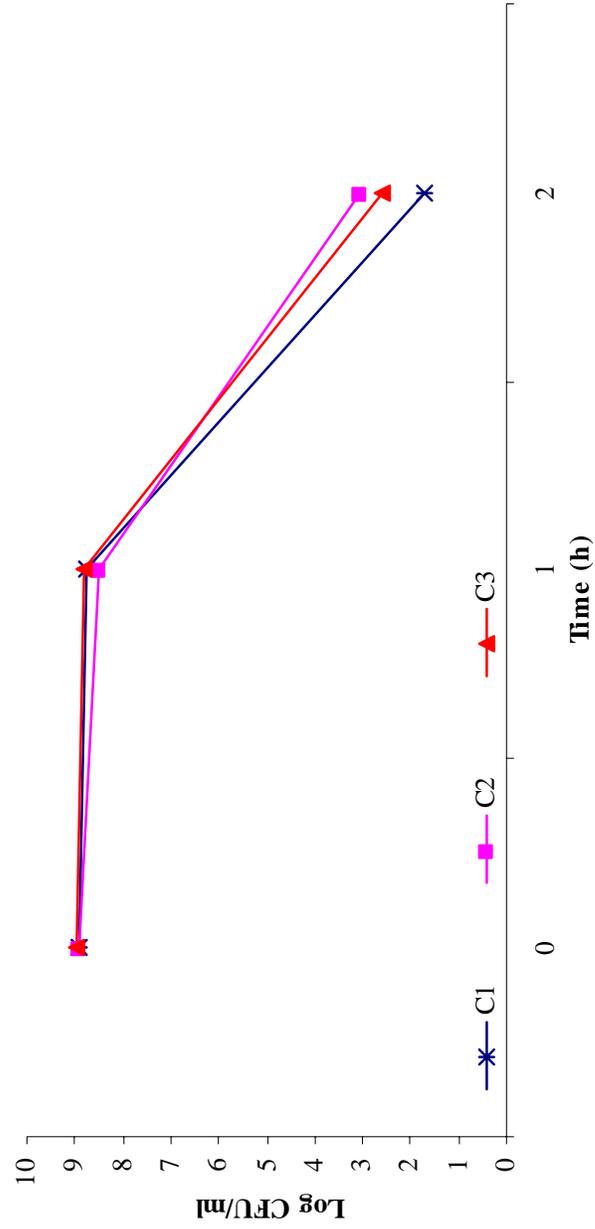


Fig. 7. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSA.

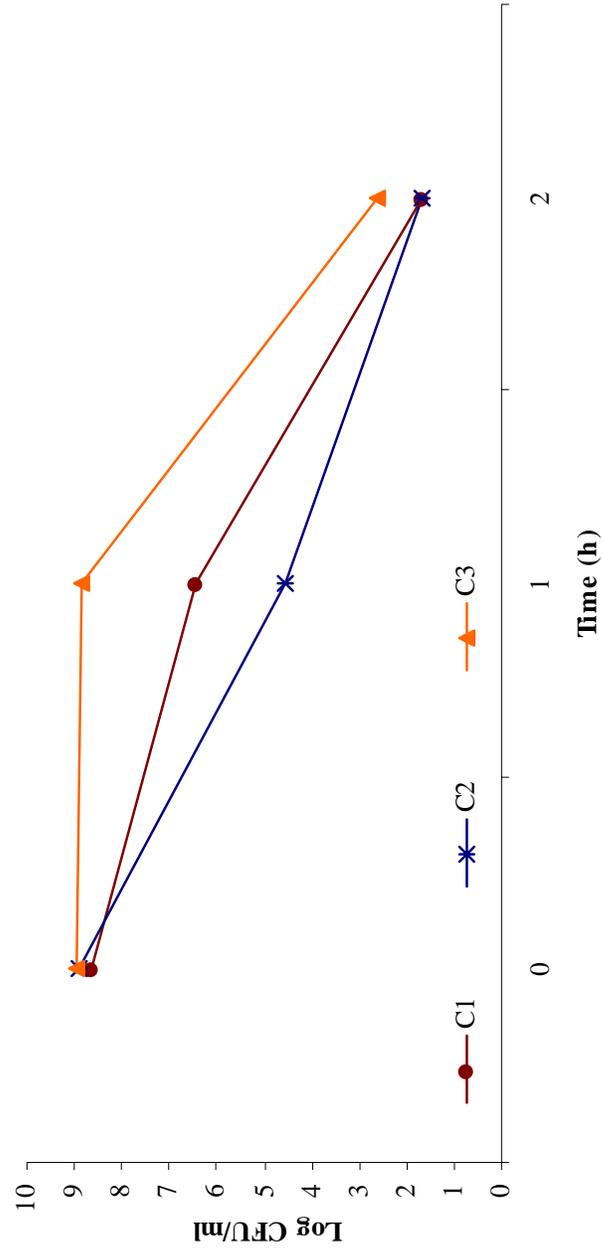


Fig. 8. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSAN.

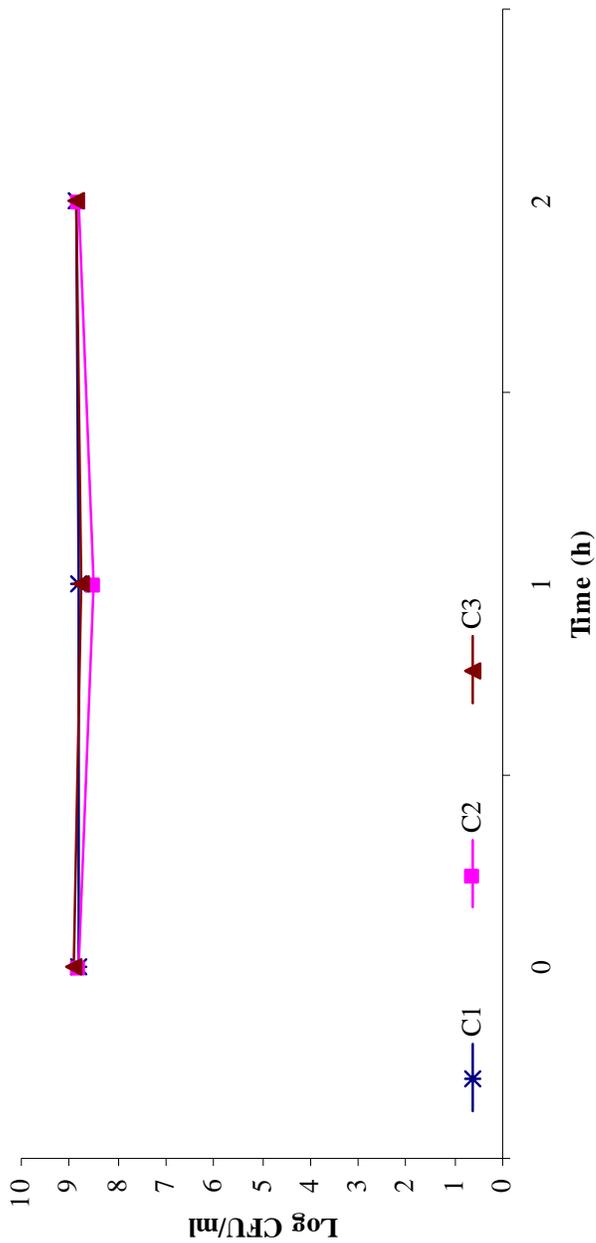


Fig. 9. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 4.0 with lactic acid and plated on TSA.

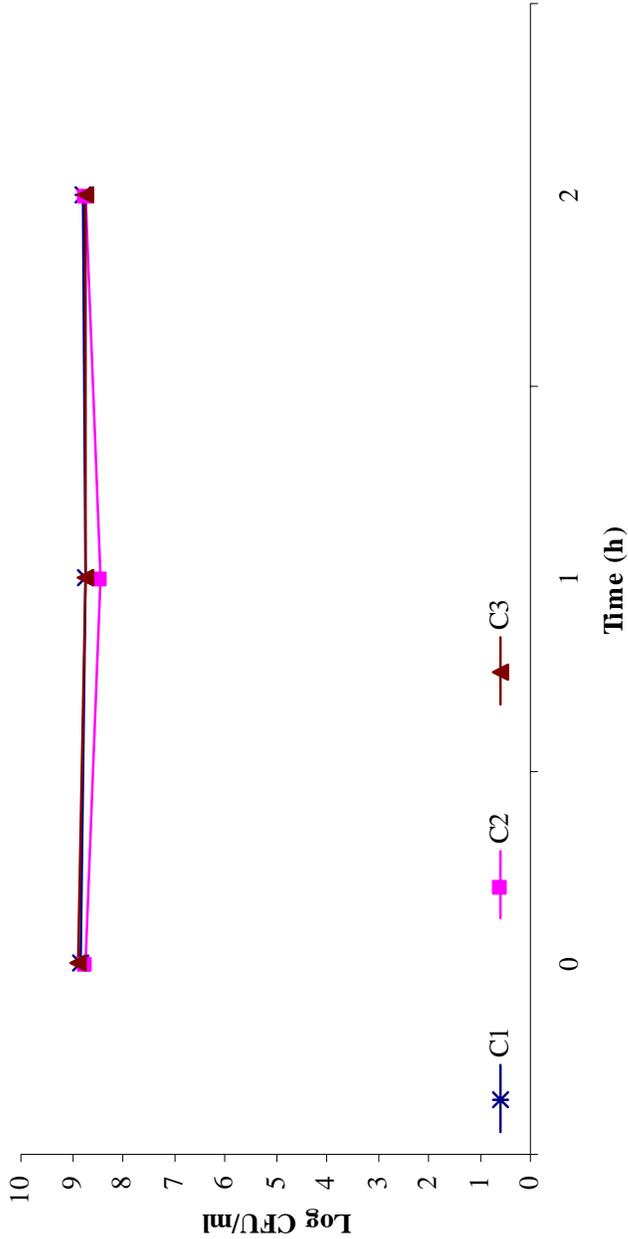


Fig.10. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 4.0 with lactic acid and plated on TSAN.

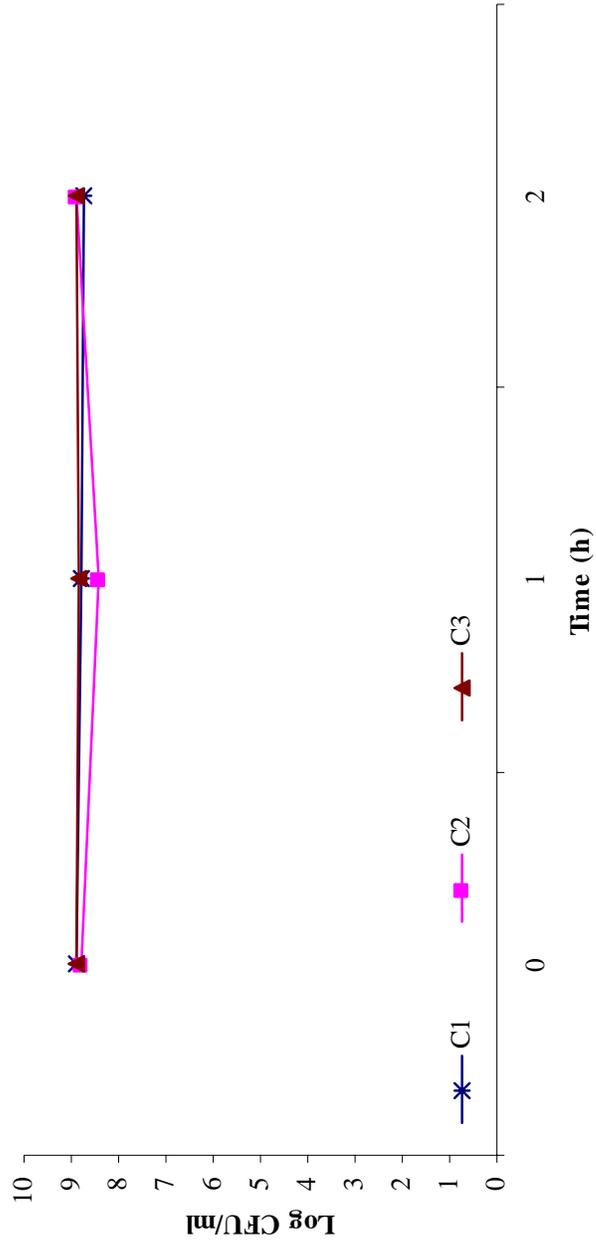


Fig. 11. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 4.0 with lactic acid and plated on TSA.

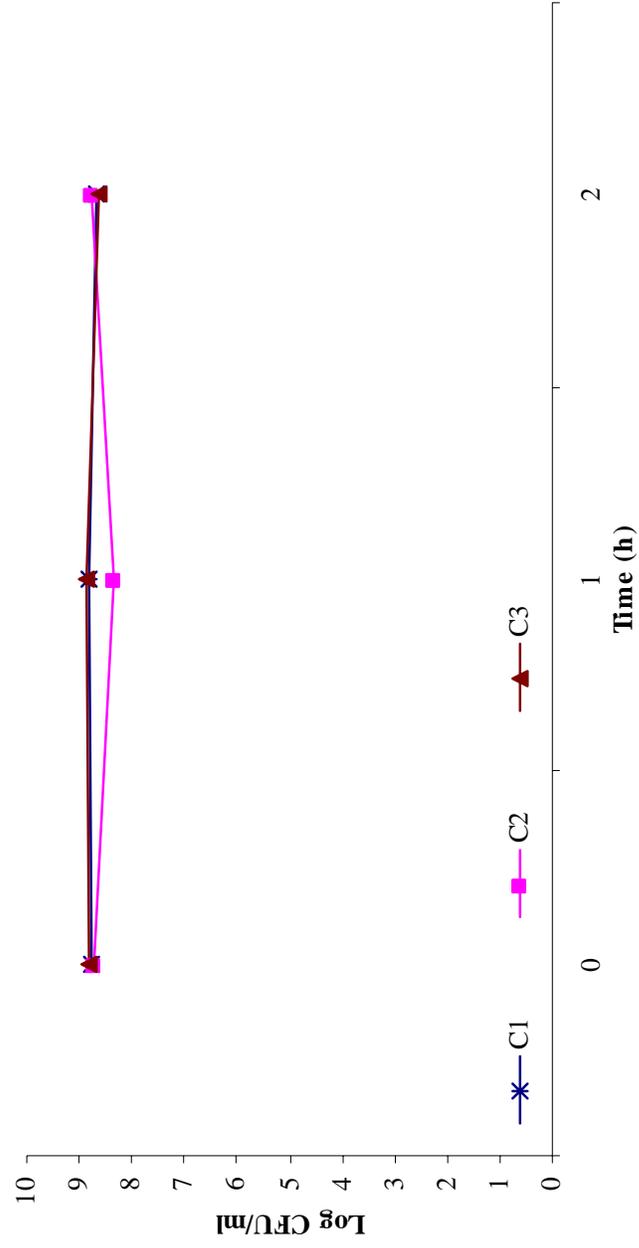


Fig.12. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 4.0 with lactic acid and plated on TSAN.

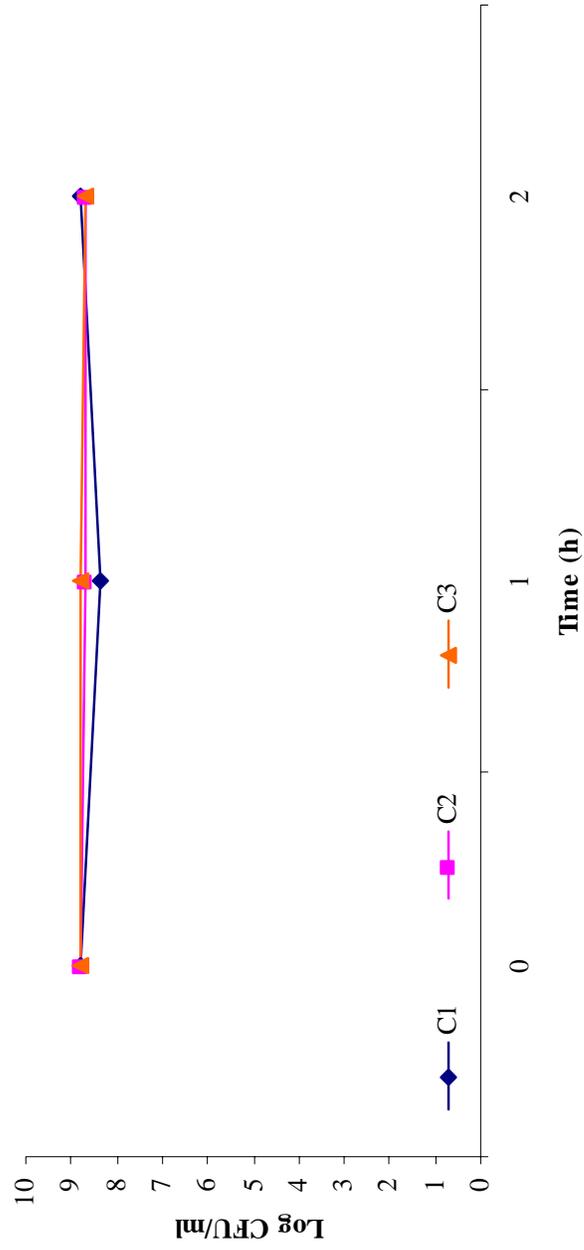


Fig. 13. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 5.0 with lactic acid and plated on TSA.

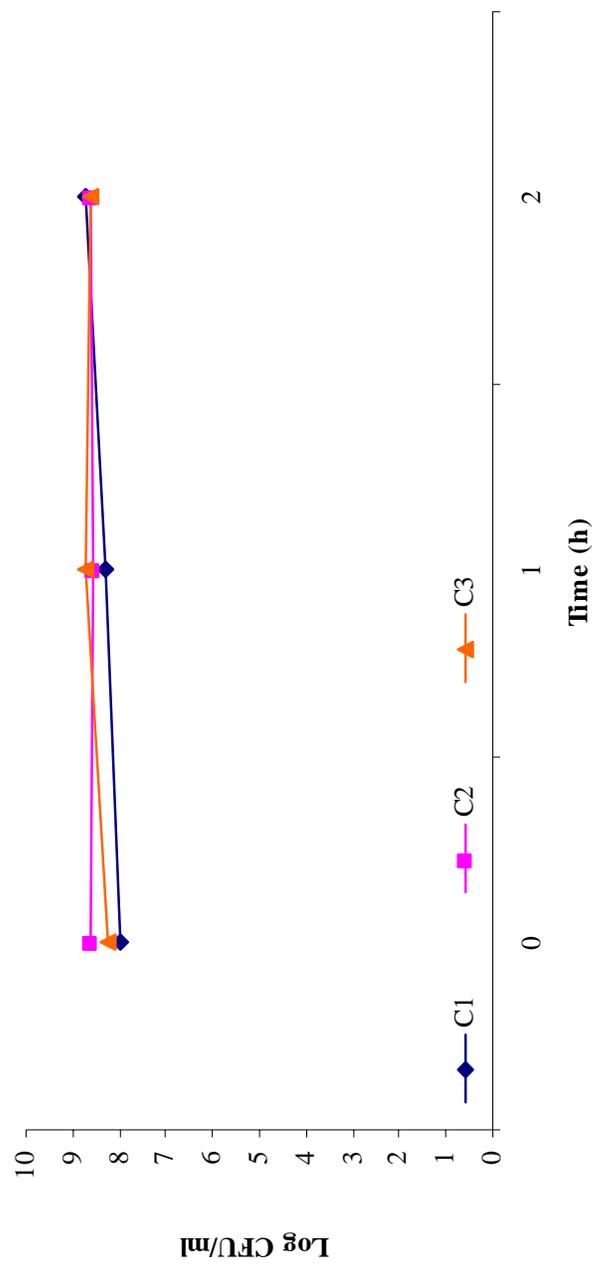


Fig. 14. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 5.0 with lactic acid and plated on TSAN.

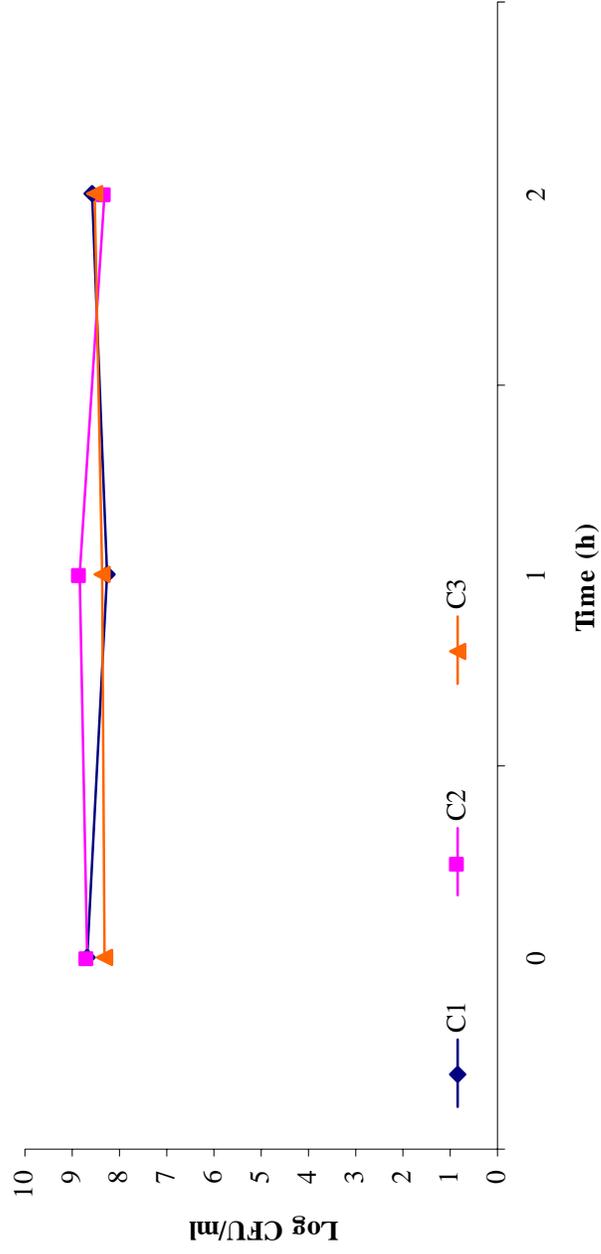


Fig. 15. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 5.0 with lactic acid and plated on TSA.

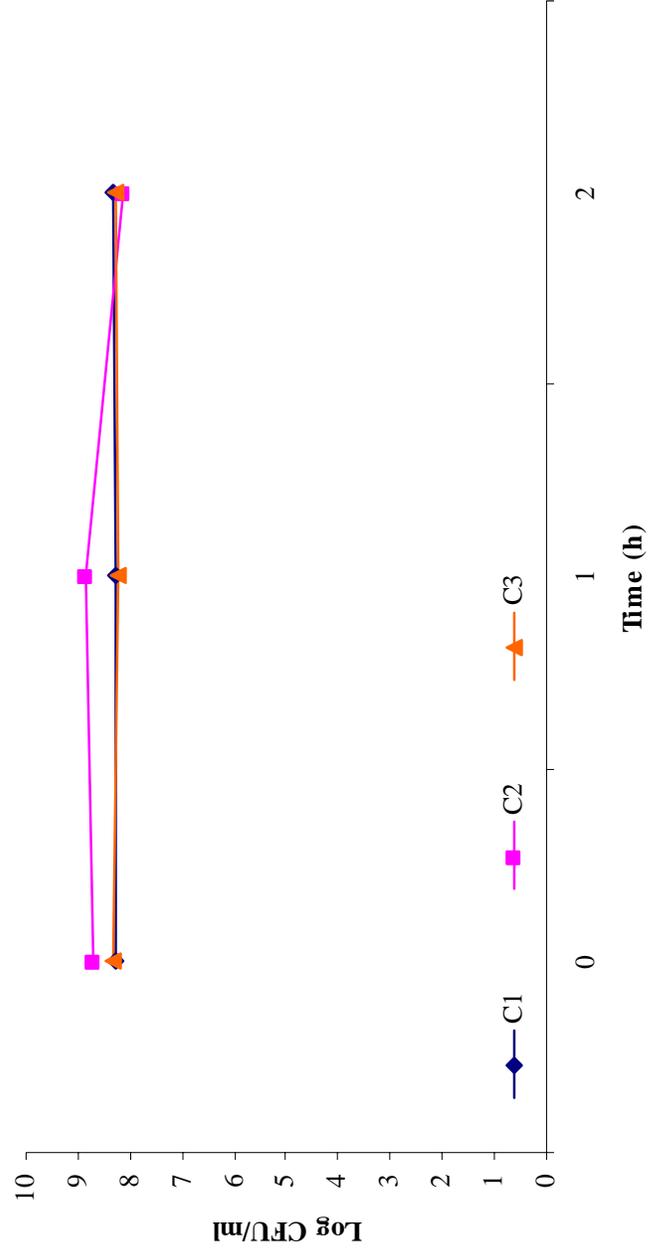


Fig. 16. Survival of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 4.0 with lactic acid and plated on TSAN.

sugar concentration resulted in an increase in heat resistance. Sublethal heat treatment of *E. coli* O157:H7 cells substantially increased their tolerance to acidity as reported by Wang and Doyle (1998). Jackson et al. (1995) found that a change in temperature resulted in a decrease in the ability of *E. coli* O157:H7 to survive heat treatment. They also compared stationary phase cultures to those in log phase grown at different temperatures (23, 30 and 37°C), and found that stationary phase cultures exhibited greater heat resistance. For this study, *E. coli* O157:H7 isolates (C1, C2, C3) were heated to 55°C and did not express differences in the TSA counts for either acid adapted or non-acid adapted conditions (Table 4). Survivor curve of *E. coli* O157:H7 isolates grown in TSB with or without glucose are showed on Figs. 17 to 20. There was evidence that the population remained constant during the heat treatment despite the conditions of cells (acid adapted or non-acid adapted) (Fig. 17 and 19).

For bacterial counts on TSAN, all isolates (C1, C2, C3) prior to heating, did not show significant differences between acid adapted and non-acid adapted conditions. Also, no significant difference in counts on TSA was reported, indicating the cells were not injured by the heat treatment at 55°C.

After the 20-min treatment there was a significant decrease in bacterial counts for all isolates (C1, C2, and C3) on TSA and TSAN for both acid adapted and non-acid adapted conditions. Decrease in counts on TSAN (Fig. 18 and 20) indicated cells were injured by the heat treatment at 55°C, and the 3 isolates (C1, C2, and C3) for either acid adapted or non-acid adapted conditions.

Microbial counts on TSA and TSAN were not significantly different (Table 5)

Table 4.
Population of *E. coli* O157:H7 under acid adapted or non-acid adapted conditions, before and after heating during 20 min at 55°C and recovered on TSA and TSAN by spread plate method.

Treatment		Counts Log CFU/ml			
		Acid adapted		Non-acid adapted	
		TSA	TSAN	TSA	TSAN
C1	Preheating ¹	8.8 ^a	8.6 ^a	8.9 ^a	8.7 ^a
C2		8.8 ^a	8.8 ^a	8.6 ^a	8.4 ^a
C3		8.9 ^a	8.9 ^a	8.9 ^a	8.9 ^a
C1	Postheating ²	8.1 ^b	5.3 ^d	7.3 ^c	5.2 ^d
C2		8.0 ^b	5.2 ^d	8.1 ^b	5.3 ^d
C3		8.3 ^b	5.6 ^d	8.0 ^b	5.0 ^d

¹Samples before start heating.

²After holding the temperature at 55°C for 20-min.

^aValues for each isolate for the entire table with the same letter (a, b, c or d) are not significantly different (P< 0.05).

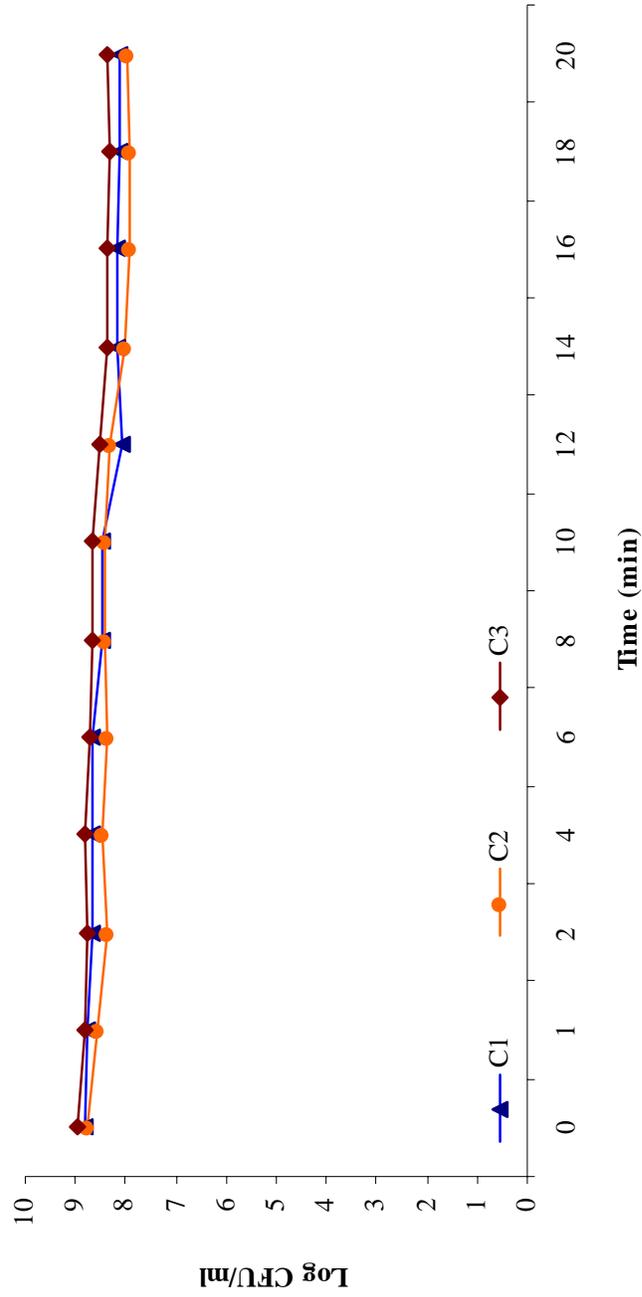


Fig. 17. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 55°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 55°C.

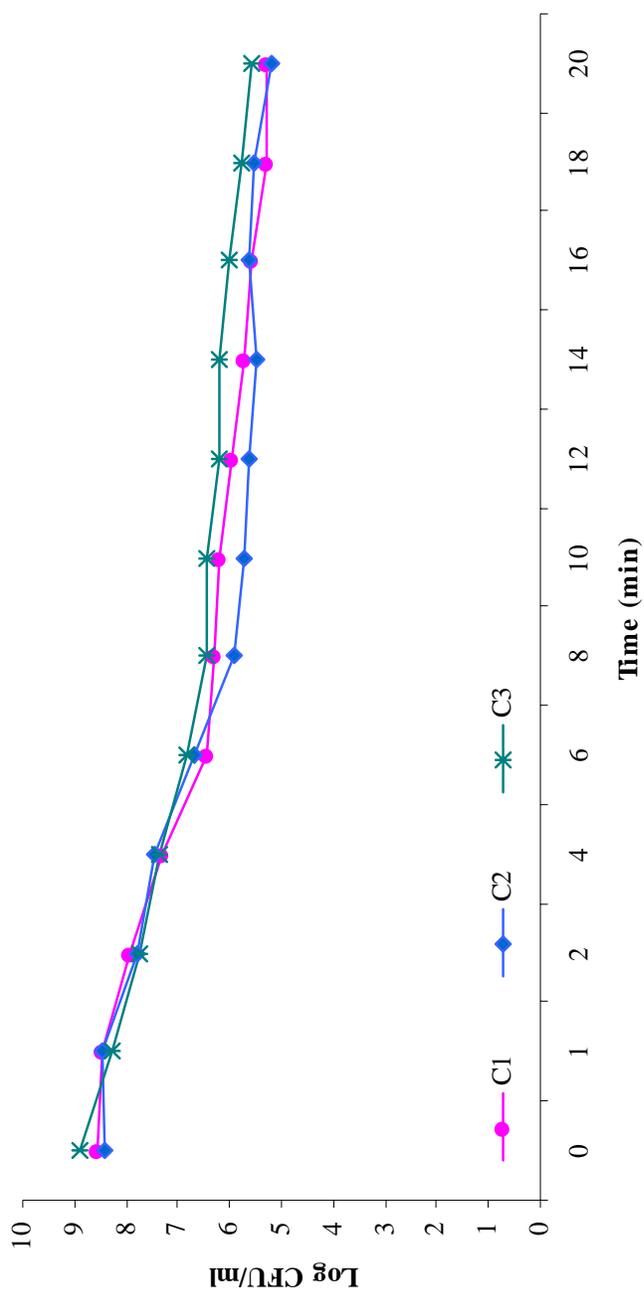


Fig. 18. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 55°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 55°C.

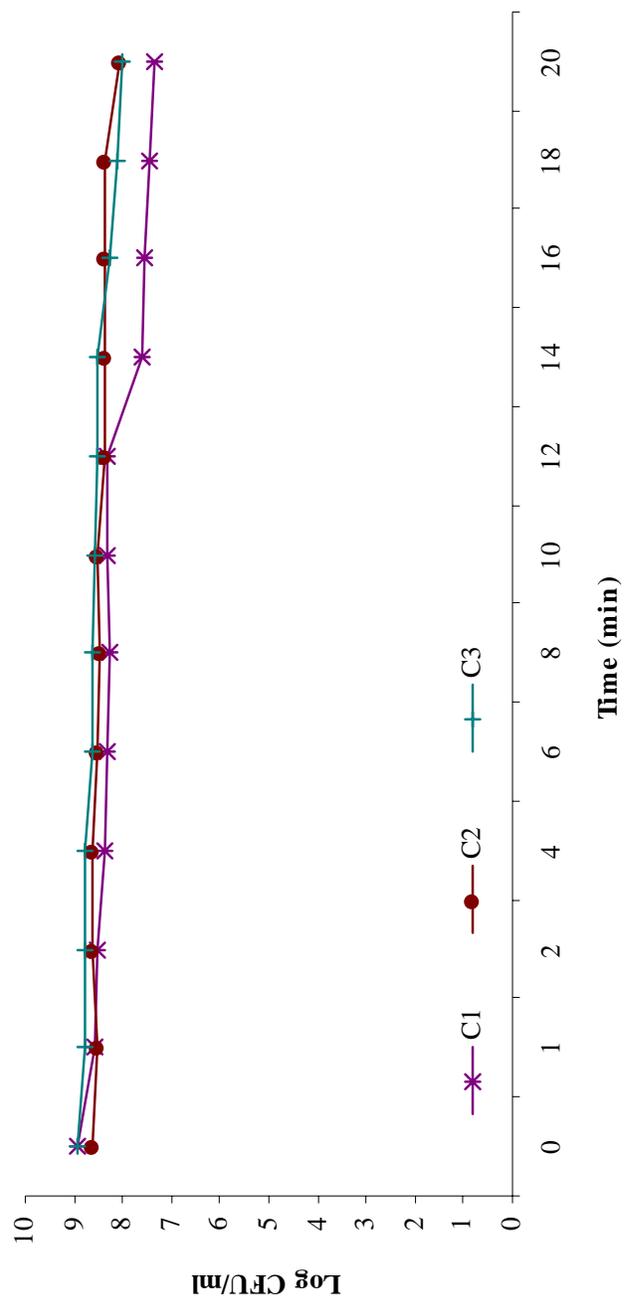


Fig. 19. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 55°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 55°C.

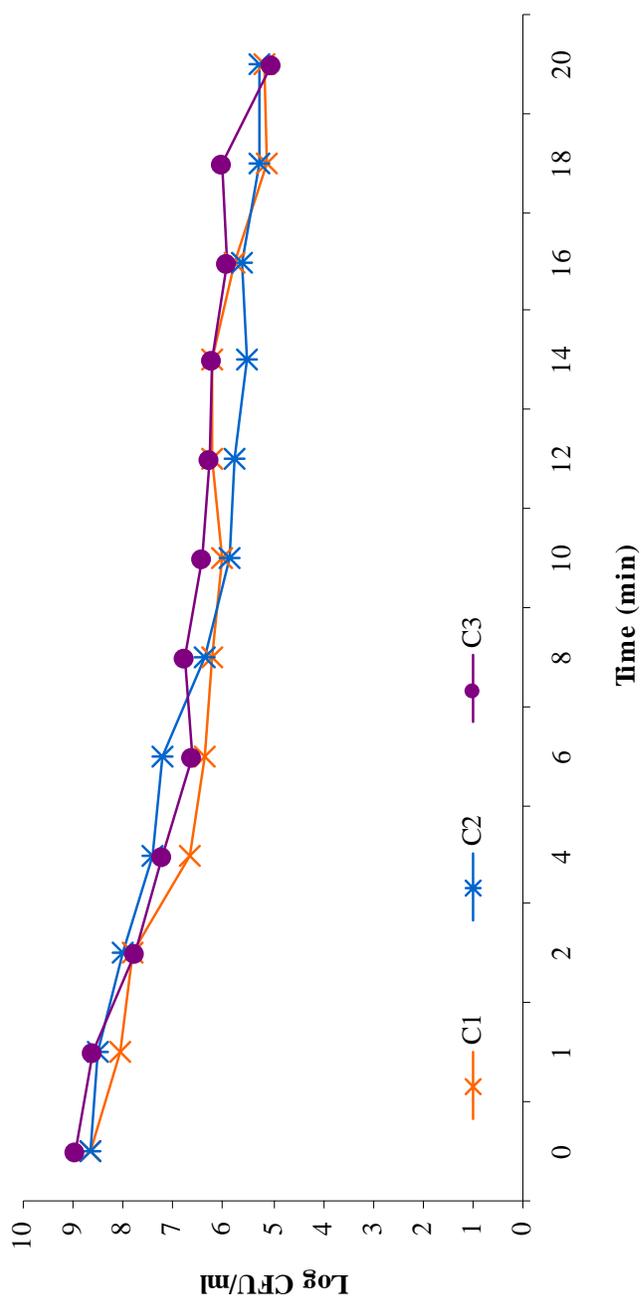


Fig. 20. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 55°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 55°C.

Table 5.
Population of *E. coli* O157:H7 under acid adapted or non-acid adapted conditions, before and after heating during 20 min at 65°C and recovered on TSA and TSAN by spread plate method.

Isolate	Treatment	Counts Log CFU/ml			
		Acid adapted		Non-acid adapted	
		TSA	TSAN	TSA	TSAN
C1	Preheating ¹	8.7 ^a	8.5 ^a	8.7 ^a	8.4 ^a
C2		8.6 ^a	8.5 ^a	8.5 ^a	8.4 ^a
C3		8.5 ^a	8.5 ^a	8.7 ^a	8.5 ^a
C1	Postheating ²	4.7 ^b	4.0 ^d	5.2 ^c	4.3 ^b
C2		5.4 ^c	5.4 ^c	4.8 ^b	4.4 ^b
C3		4.6 ^b	4.5 ^b	3.9 ^d	3.0 ^e

¹Samples before start heating.

²After holding the temperature at 65°C for 20-min.

^aValues for each isolate for the entire table with the same letter (a, b, c, d or e) are not significantly different (P< 0.05).

for the three isolates (C1, C2, and C3) under acid adapted and non acid adapted conditions, before the heat treatment at 65°C. Survivor curve of *E. coli* O157:H7 isolates grown in TSB with or without glucose are shown in Figs. 21 to 24. Bacterial counts on TSA decreased after heat treatment, indicating cells were injured (Fig. 21 and 23). The 3 isolates C1, C2, and C3, were significantly different after heat treatment for both conditions (acid adapted and non-acid adapted). C1 and C3 isolates were significantly different than isolate C2 at acid adapted conditions, but, all isolates were significantly different under non-acid adapted conditions when they were plated on TSA.

Isolate C1 plated on TSAN after heat treatment showed a significant difference compared to isolates C2 and C3 under acid adapted conditions. However, under non-acid adapted conditions isolate C2 was not significantly different after heat treatment compared with isolates C1 and C3 which were significantly different (Fig. 22 and 24). Bacterial counts on TSAN for heat treatment at 65°C was similar to that at 55°C in which the bacterial count decreased indicating that cells were injured, and not able to recover.

Comparison of the heat resistance at 55°C and 65°C indicated differences in microbial reduction when both acid adapted and non-acid adapted isolates were plated on TSA. At 65°C log reduction ranged from 3.2 log-4.8 log; whereas, only 0.8 log reductions were detected at 55°C.

For purpose of this study, extreme parameters such as pH 3.0 for acid sensitivity and 65°C for heat resistance under the same acid adapted and non-acid adapted conditions were taken for further characterization of the “ideal” indicator bacteria to

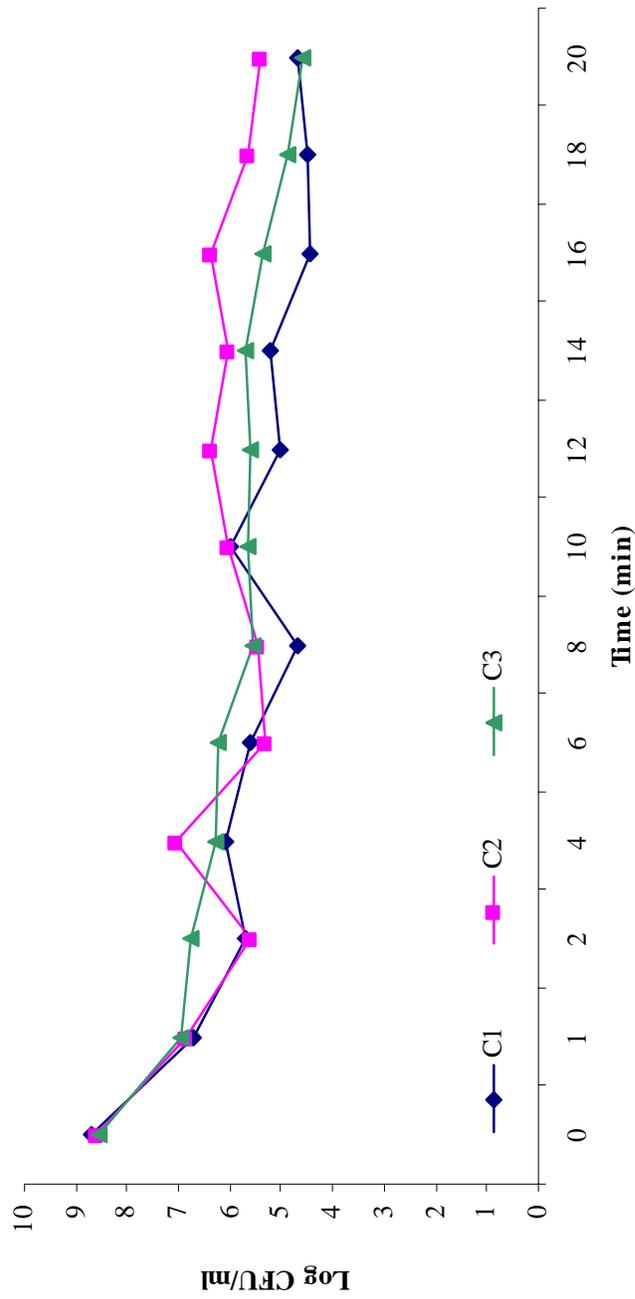


Fig. 21. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

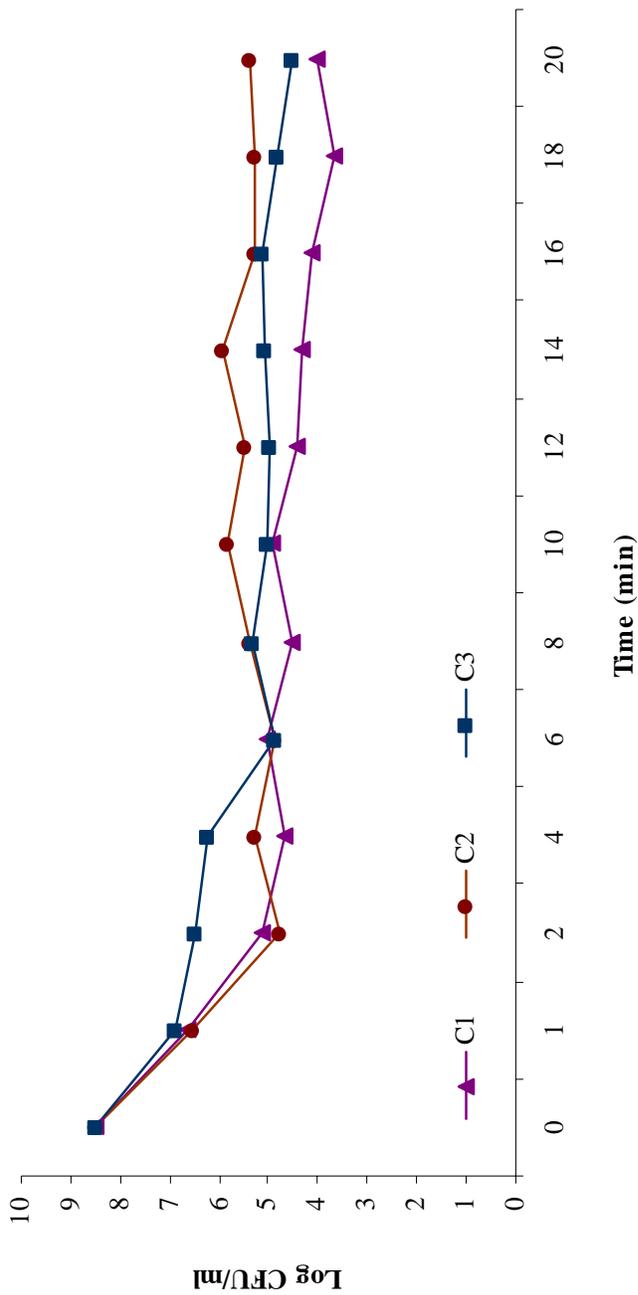


Fig. 22. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

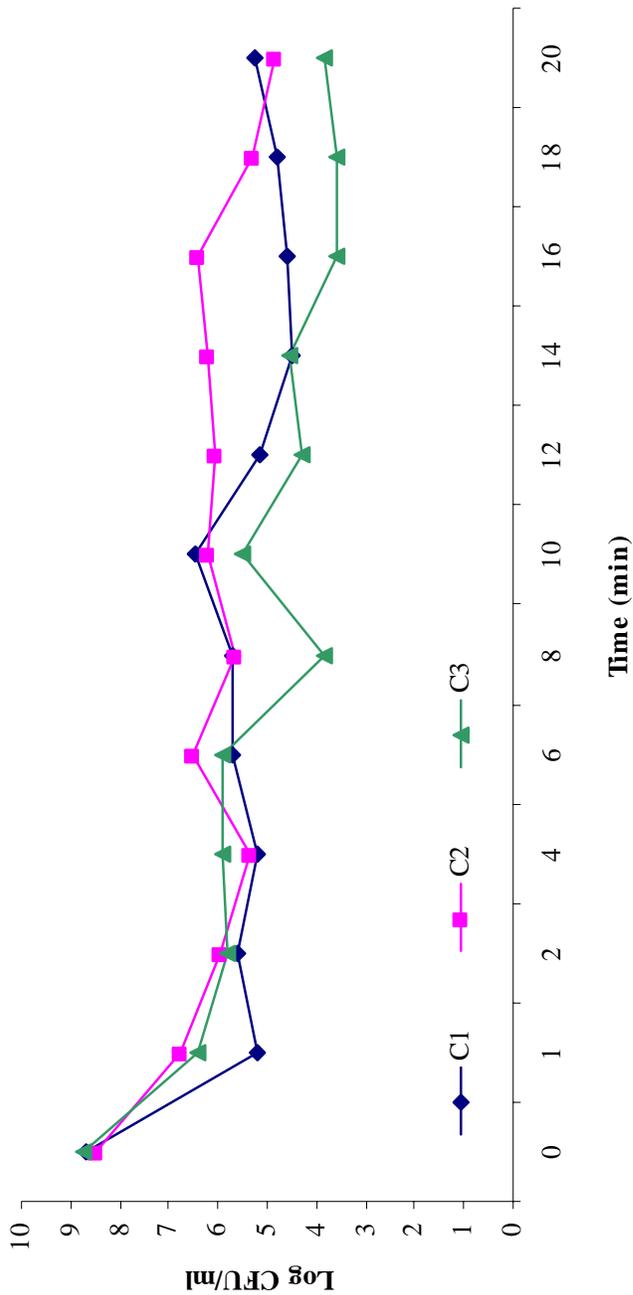


Fig. 23. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

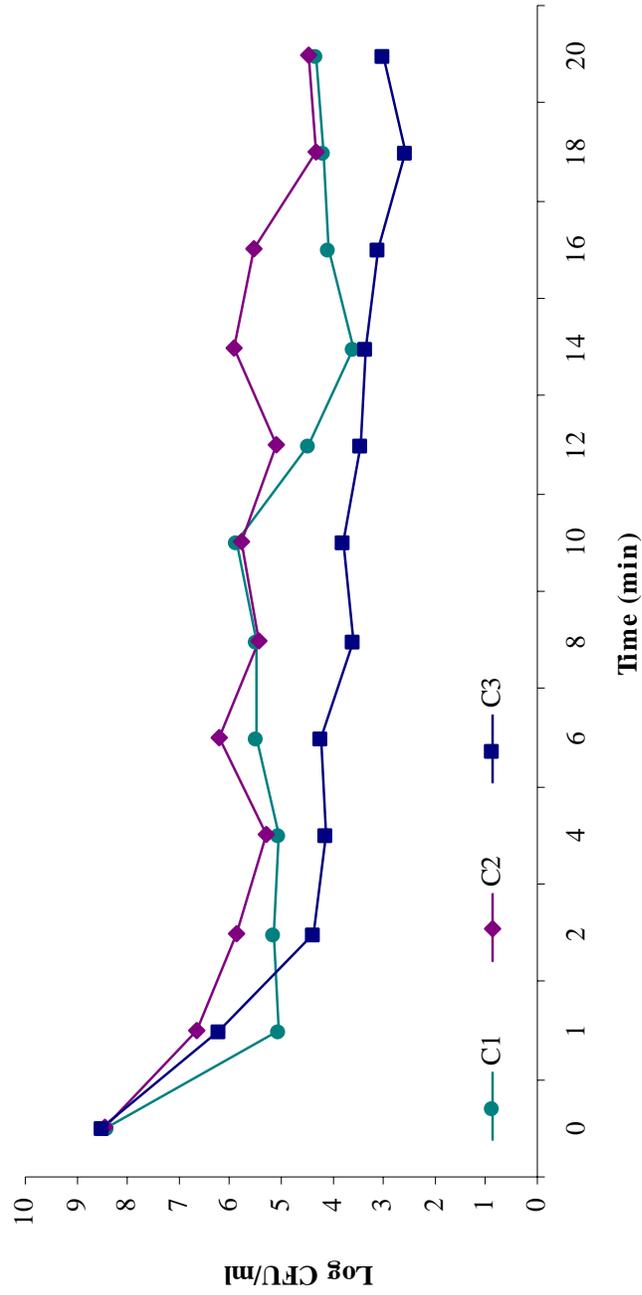


Fig. 24. Survivor curve of *E. coli* O157:H7 isolates (C1, C2, and C3) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

determine their similarity with *E. coli* O157:H7 isolated from hides.

Indicators

Indicator organisms can help to provide information about process failure, post-processing contamination, contamination from the environment, and the general level of hygiene under which the food was processed and stored (Doyle et al., 2001). Vold et al. (2000) found that using measures for reducing pathogens also reduced the background microflora. A suitable indicator organism needed to be identified that grew in the same environment as *E. coli* O157:H7 as well as had the same acid and heat resistance properties.

Jay (2000) suggested that the ideal indicator used to assess food safety should be easily and rapidly distinguishable. Also, the bacteria should have a history of constant association with the pathogen whose presence it is to indicate, and it should always be present when the pathogen of concern is present. The indicator organism numbers should be correlated with those of the pathogens, have a die-off rate that at least parallels that of the pathogen. It is advantageous for indicators to persist slightly longer than the pathogen of concern and be absent from foods that are free of the pathogen except perhaps at certain minimum numbers.

The original microflora in products may vary from one to another. The microflora present in a particular batch for a particular product depends upon the source of contamination and the respective environment, such as storage temperature, storage atmosphere, transportation and handling processes. On the other hand, heat resistance or

acid resistance of the original microflora would vary depending of these sources of contamination. The genus and species of bacteria that usually predominate are those that can most easily utilize the nutrients present (Juneja, 2003).

Acid resistance of potential indicators

Preliminary screening on TSA at pH 4.5 and 5.0 indicated acid resistance in some microorganisms isolated from hide samples. Those identified organisms were *E. coli* Type I, confirmed by VITEK.

After potential indicators were identified, acid resistance at pH 3.0 and heat resistance at 65°C were determined. None of the 10 potential indicator bacteria survived those parameters. Eight potential indicator bacteria were supplied by Iowa State University (Ames, IA) through their contribution in this collaborative study. Upon receipt, indicators were analyzed under the established parameters (acid resistant at pH 3.0 and heat resistant at 65°C). Those potential indicators were confirmed to be *E. coli* Type I using VITEK.

The 8 potential indicators were tested at pH 3.0 for acid resistance and only 5 survived for 2 h. Those 5 isolates (C4, C5, C6, C7, and C8) were not significantly different when plated on TSA and all presented similar trends (Figs. 25 to 28). Isolates C7, and C8 under acid adapted conditions were similar to *E. coli* O157:H7 isolates (C2 and C3), while isolates C4, C5, and C6 presented significant differences compared with C1, C2, and C3 isolates. Under non-acid adapted conditions, isolates C6, C7, and C8,

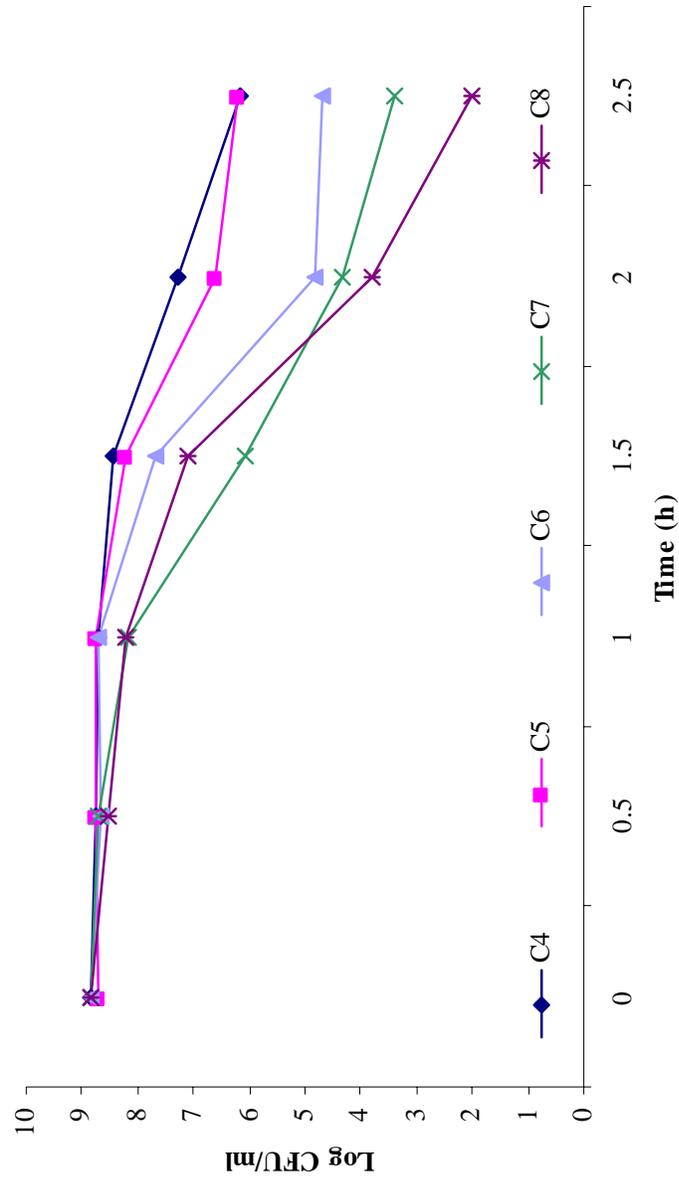


Fig. 25. Survival of potential indicator bacteria isolates (C4, C5, C6, C7, and C8) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSA.

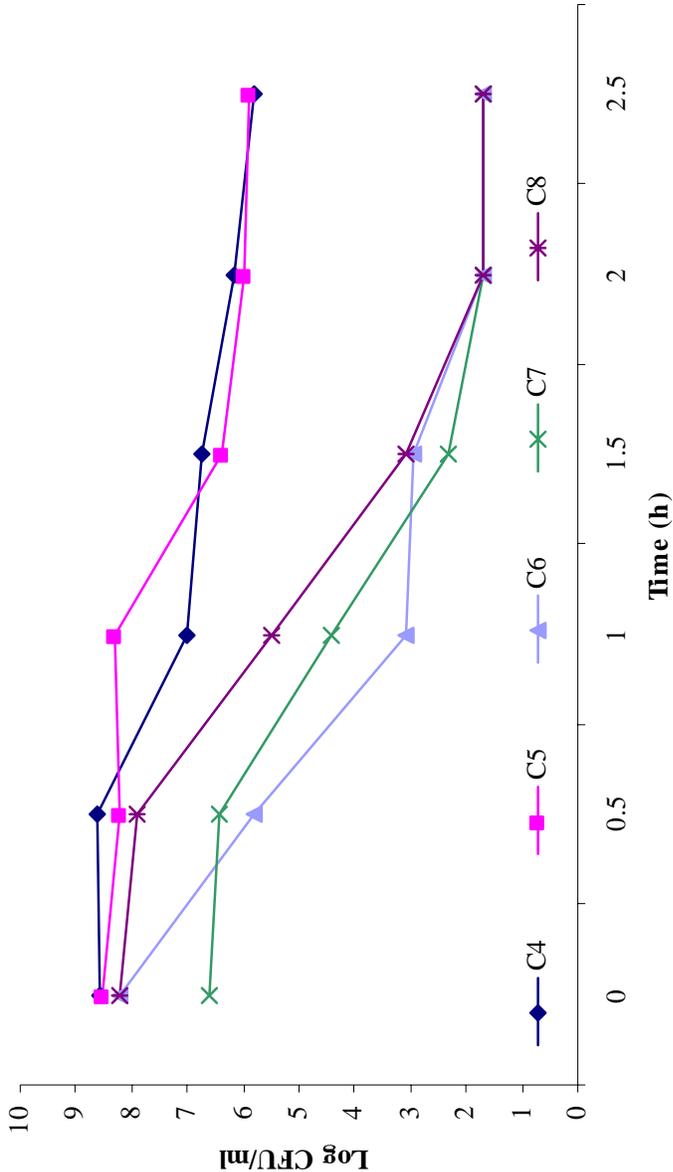


Fig. 26. Survival of potential indicator bacteria isolates (C4, C5, C6, C7, and C8) grown in TSB (37°C, 18 h) with no glucose (non-acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSAN.

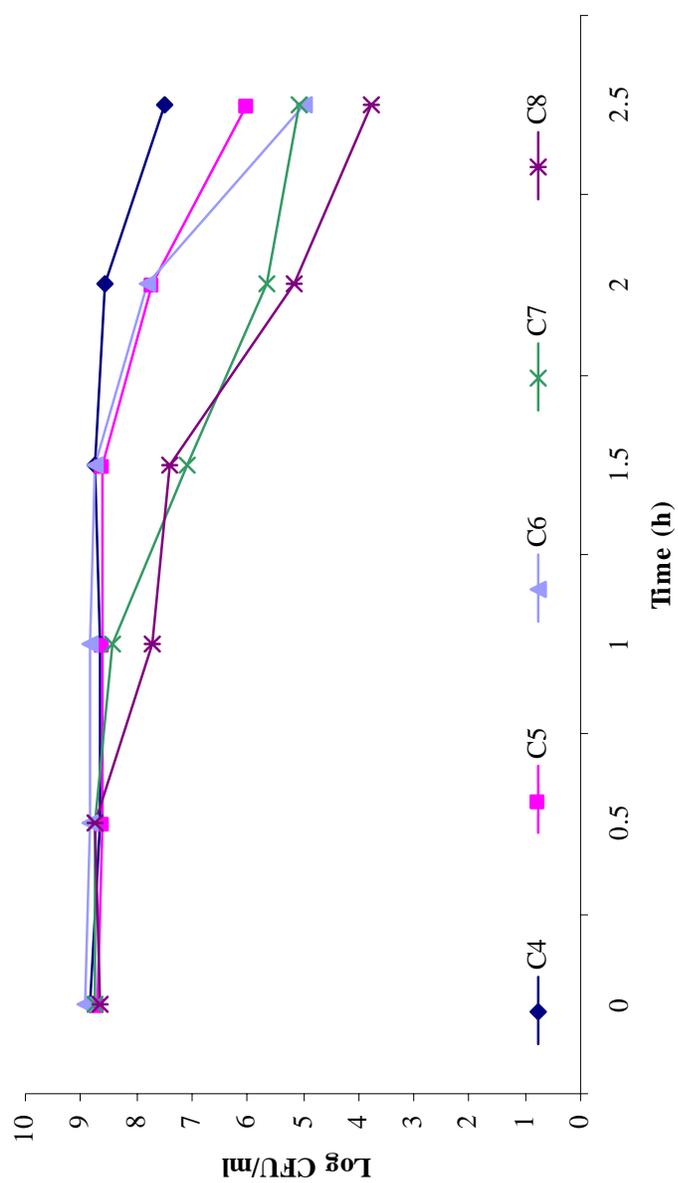


Fig. 27. Survival of potential indicator bacteria isolates (C4, C5, C6, C7, and C8) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSA.

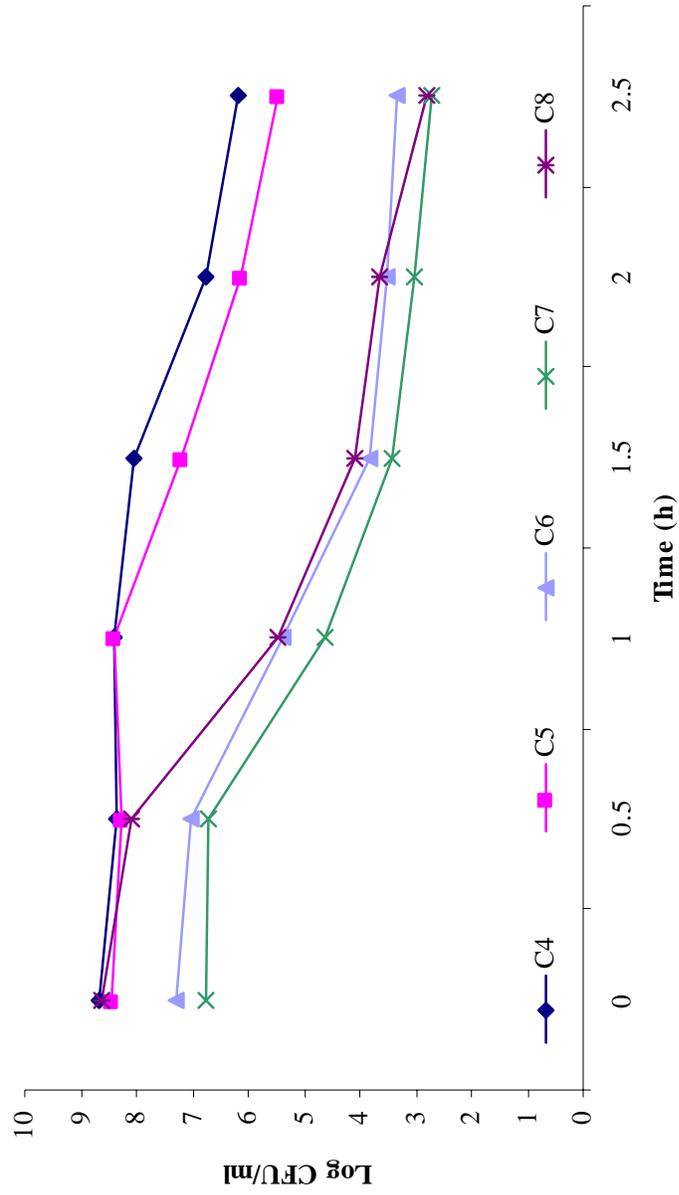


Fig. 28. Survival of potential indicator bacteria isolates (C4, C5, C6, C7, and C8) grown in TSB (37°C, 18 h) with 1% glucose (acid adapted) following growth (37°C, 2 h) in acidified PBS adjusted to pH 3.0 with lactic acid and plated on TSAN.

were not significantly different than *E. coli* O157:H7 isolates (C1, C2, and C3). Overall, all potential indicators plated on TSA or TSAN were not significantly different for either acid adapted or non-acid adapted conditions.

The decrease in populations of potential indicators plated on TSA and under non-acid adapted conditions were 2.6, 2.5, 4.2, 5.4, and 6.8 log for C4, C5, C6, C7, and C8, respectively. Corresponding values obtained under acid adapted conditions were 1.3, 2.7, 3.9, 3.7 and 4.9 log for C4, C5, C6, C7, and C8, respectively.

Injury was observed for all potential indicators under non-acid adapted conditions when plated on TSAN. For isolates C4 and C5 the injury was less when compared to isolates C6, C7, and C8, which did not present any growth after 2-h incubations (Table 6). Cells grown in acidified media exhibited a decreased tolerance to sodium chloride (NaCl), indicating there was injury due to exposure to the low pH. More injury was observed in non-acid adapted cells than in acid adapted cells, indicated by the inability of large numbers of non-acid adapted cells to form colonies on TSAN compared to TSA. Potential indicators, in general, were more resistant than those of *E. coli* O157:H7 isolates.

Heat resistance of potential indicators

Juneja, et al. (2003) reported on the relationship between the cell's ability to grow and multiply and the cell's ability to survive adverse environmental effects, such as heat. This study also implied that a more heat resistant cell might also be a cell with a faster growth rate. The mechanism that enhances a cell's ability to resist and stay viable

Table 6.

Population of *E. coli* O157:H7 isolates (C1, C2, and C3) and potential indicators (C4, C5, C6, C7, and C8) under acid adapted or non-acid adapted conditions, pre and post acid treatment during 2.5 h at pH 3.0 and recovered on TSA and TSAN by spread plate method.

Isolate	Treatment	Counts Log CFU/ml			
		Acid adapted		Non-acid adapted	
		TSA	TSAN	TSA	TSAN
C1	Pre-acid ¹	8.9 ^a	8.6 ^a	8.9 ^a	8.8 ^a
C2		8.9 ^a	8.9 ^a	8.9 ^a	8.8 ^a
C3		8.9 ^a	8.9 ^a	8.9 ^a	8.9 ^a
C4		8.8 ^a	8.7 ^a	8.8 ^a	8.6 ^a
C5		8.7 ^a	8.4 ^a	8.7 ^a	8.5 ^a
C6		8.9 ^a	7.3 ^b	8.9 ^a	8.2 ^a
C7		8.8 ^a	6.8 ^b	8.8 ^a	6.6 ^b
C8		8.7 ^a	8.6 ^a	8.8 ^a	8.2 ^a
C1	Post-acid ²	<1.7 ^g	<1.7 ^g	<1.7 ^g	<1.7 ^g
C2		2.6 ^f	<1.7 ^g	<1.7 ^g	<1.7 ^g
C3		<1.7 ^g	<1.7 ^g	<1.7 ^g	<1.7 ^g
C4		7.5 ^b	6.2 ^c	6.2 ^c	5.8 ^c
C5		6.0 ^c	5.4 ^d	6.2 ^c	5.9 ^c
C6		5.0 ^d	3.3 ^{ef}	4.7 ^d	<1.7 ^g
C7		5.1 ^d	2.7 ^f	3.4 ^e	<1.7 ^g
C8		3.8 ^e	2.8 ^f	2.0 ^g	<1.7 ^g

¹Samples at initial contact with PBS at pH 3.0.

²After 2.5 h incubation at 37°C at pH 3.0.

^aValues for each isolated with the same letter (a, b, c, d, e, f, or g) are not significantly different (P < 0.05).

may hinder or at least not contribute to its ability to grow and multiply.

All *E. coli* O157:H7 isolates and potential indicator isolates were not significantly different before heat treatment for both acid adapted and non-acid adapted conditions when plated on TSA or TSAN. Heat treatment at 65°C resulted in significant numbers of injured cells for both *E. coli* O157:H7 and potential indicator isolates. Those differences or similarities are shown in Table 7.

Potential indicator isolates showed similar trends when plated on TSA under either acid adapted or non-acid adapted conditions, but those trends were different when plated on TSAN (Figs. 29 to 32), in both cases, reduction was observed. The log reduction for C1, C2, and C3 isolates under acid adapted conditions was 4.0, 3.2 and 3.9 respectively. For potential indicator isolates C4, C5, C6, C7, and C8 reduction total of 5.4, 4.0, 2.4, 4.2, and 4.6 log, respectively. Isolate C5 had a similar reduction (4.0 log) to *E. coli* O157:H7 isolates while C6 (2.4 log) had a lower reduction indicating more resistance to heat treatment at 65°C under acid adapted conditions.

Under non-acid adapted conditions the population of potential indicator isolates decreased (4.3, 4.6, 2.4, 6.8, and 6.1 log for C4, C5, C6, C7, and C8, respectively). Potential indicator isolate C5 had a similar reduction to *E. coli* O157:H7 isolates. Isolate C6 was more resistant to heat treatment under non-acid adapted conditions. Both isolates C5 and C6 were similar under acid adapted and non-acid adapted conditions.

Isolates C4 and C8 recovered on TSAN under acid adapted conditions were more susceptible to heat treatment after 20 min at 65°C. Isolates C5, C6, and C7 recovered better on TSAN, indicating more resistance to the heat treatment. Under non-acid

Table 7.

Population of *E. coli* O157:H7 isolates (C1, C2, and C3) and potential indicators (C4, C5, C6, C7, and C8) under acid adapted or non-acid adapted conditions, before and after heating during 20 min at 65°C and recovered on TSA and TSAN by spread plate method

Isolate	Treatment	Counts Log CFU/ml			
		Acid adapted		Non-acid adapted	
		TSA	TSAN	TSA	TSAN
C1	Preheating ¹	8.7 ^a	8.5 ^a	8.7 ^a	8.4 ^a
C2		8.6 ^a	8.5 ^a	8.5 ^a	8.4 ^a
C3		8.5 ^a	8.5 ^a	8.7 ^a	8.5 ^a
C4		8.7 ^a	8.7 ^a	8.7 ^a	8.7 ^a
C5		8.7 ^a	8.7 ^a	8.7 ^a	8.7 ^a
C6		8.7 ^a	8.7 ^a	8.8 ^a	8.7 ^a
C7		8.8 ^a	8.7 ^a	8.5 ^a	8.5 ^a
C8		8.9 ^a	8.8 ^a	8.8 ^a	8.7 ^a
C1	Postheating ²	4.7 ^b	4.0 ^d	5.2 ^c	4.3 ^b
C2		5.4 ^c	5.4 ^c	4.8 ^b	4.4 ^b
C3		4.6 ^b	4.5 ^b	3.9 ^d	3.0 ^e
C4		3.3 ^e	<1.7 ^f	4.4 ^b	4.1 ^b
C5		4.7 ^b	4.4 ^b	4.1 ^b	3.5 ^e
C6		6.3 ^g	4.1 ^b	6.3 ^g	4.1 ^b
C7		4.6 ^b	<1.7 ^f	4.7 ^b	2.3 ^e
C8		4.3 ^b	3.7 ^d	2.7 ^e	<1.7 ^f

¹Samples before start heating

²After holding the temperature at 65°C for 20-min

^aValues for each isolated with the same letter (a, b, c, d, e, f or g) are not significantly different (P < 0.05).

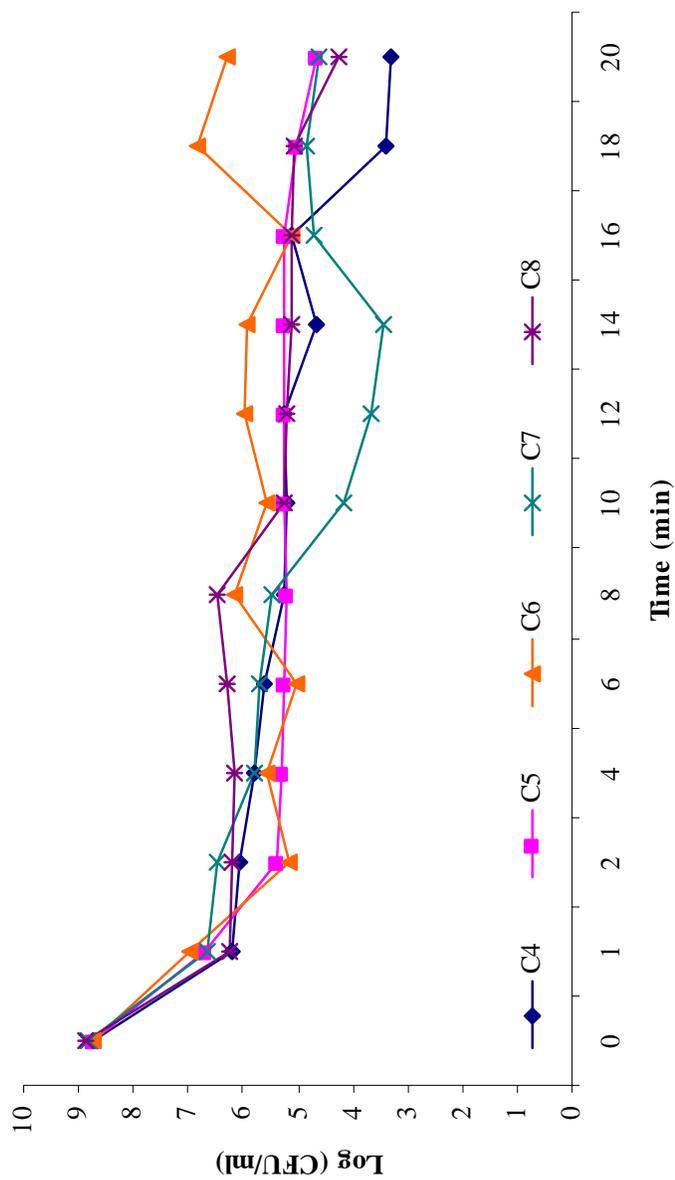


Fig. 29. Survivor curve of potential indicator isolates (C4, C5, C6, C7, and C8) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

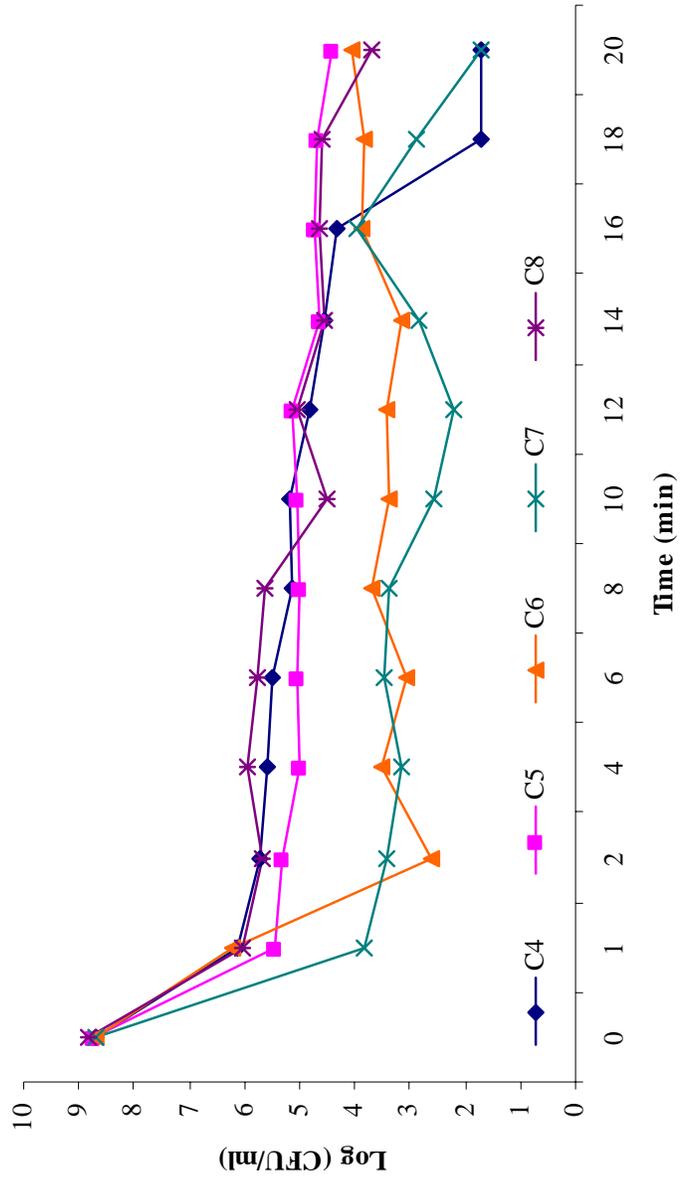


Fig. 30. Survivor curve of potential indicator isolates (C4, C5, C6, C7, and C8) grown in TSB with 1% glucose (acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

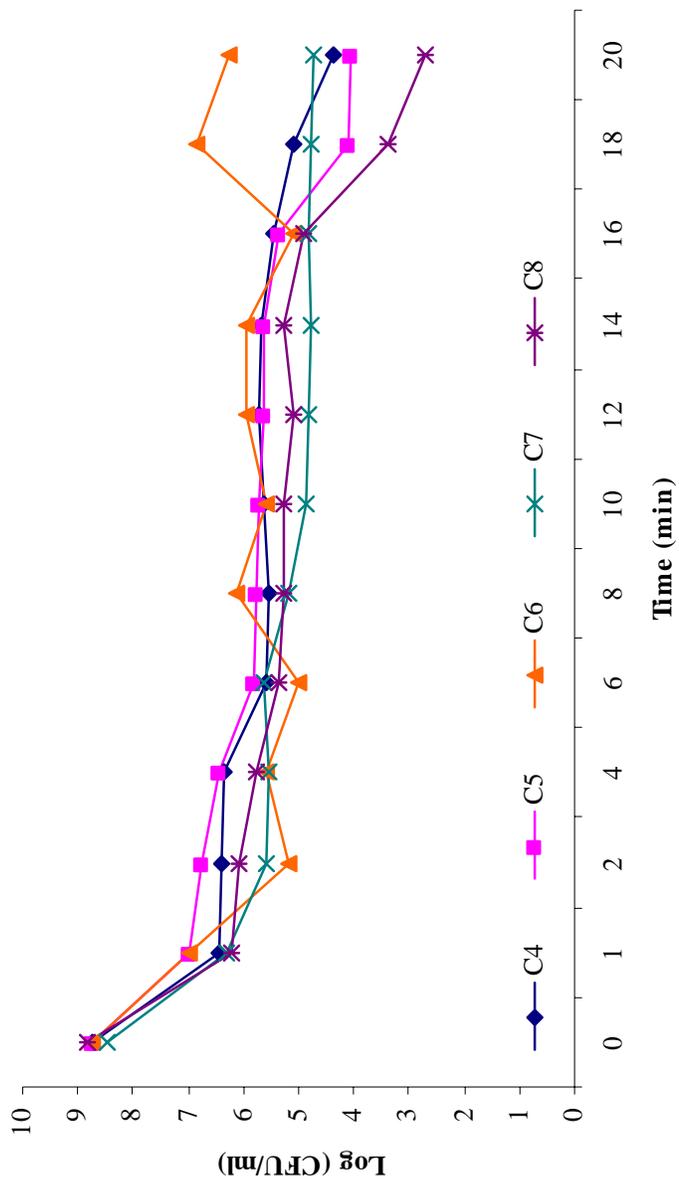


Fig. 31. Survivor curve of potential indicator isolates (C4, C5, C6, C7, and C8) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSA. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

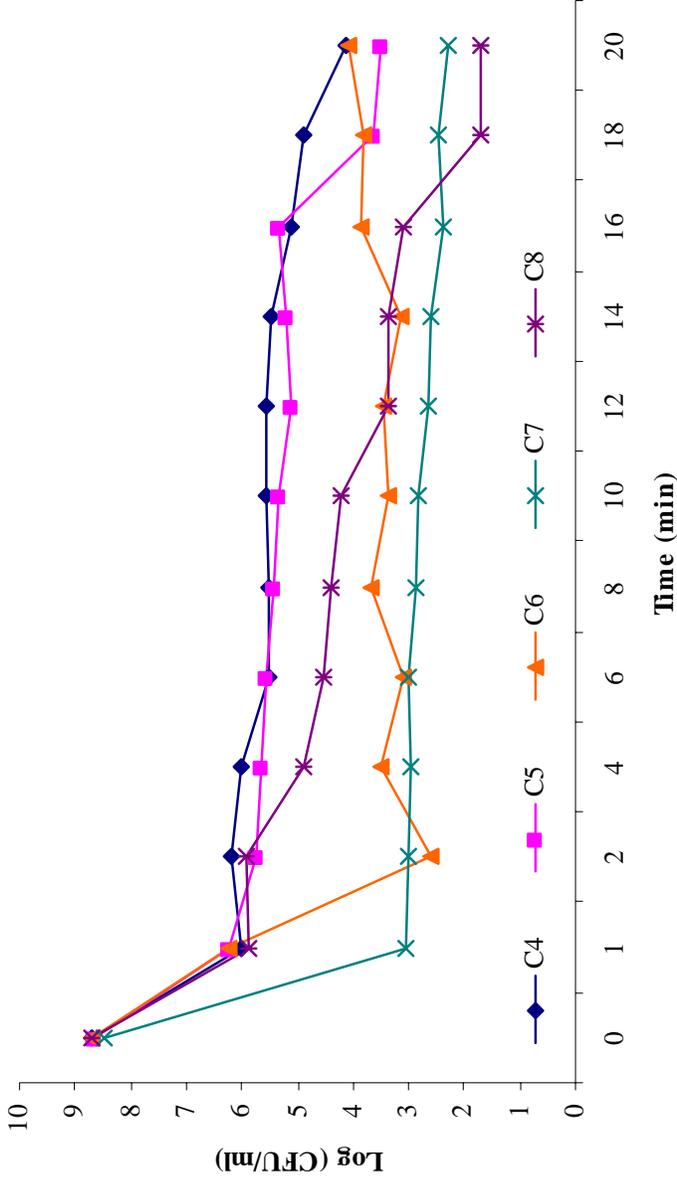


Fig. 32. Survivor curve of potential indicator isolates (C4, C5, C6, C7, and C8) grown in TSB with no glucose (non-acid adapted) at 37°C, 18 h; following growth in PBS held at 65°C for 20 min, harvested every 2 min and plated on TSAN. Time 0 represents the population before heat treatment; time 1 represents the population immediately after reaching 65°C.

adapted conditions, potential indicator C8 was not able to recover from the treatment.

Isolates C4 and C5 showed greater recovery therefore greater resistance.

CONCLUSIONS

Microbiological detection of foodborne pathogens is not effective for monitoring critical control points (CCP) within a slaughter/processing Hazard Analysis and Critical Control Points (HACCP) plan. In addition, pathogens are usually absent from carcass surface samples and uneven distribution makes it difficult to obtain a representative sample. With proper data collection, the reduction of an indicator bacteria or group at a point in processing can indicate that a specific pathogen is being effectively controlled. Since *E. coli* O157:H7 has been shown to have some heat and acid resistance, typical indicator organisms may not accurately predict the reduction of this pathogen by carcass decontamination procedures. However, obtaining potential indicator bacteria from the same environmental reservoir as *E. coli* O157:H7, may provide non-pathogenic indicators with similar heat- and acid-resistance characteristics suitable for use in processing plant environments for validation and verification of carcass decontamination treatments within HACCP plans.

Potential indicator bacteria were isolated from hides of cattle at 8 slaughter facilities in Arizona, Georgia, and Texas, and compared with strains of *E. coli* O157:H7 isolated from the same locations to determine similarity in acid- and heat-resistance characteristics. After testing heat resistance at 2 heating temperatures (55 and 65°C) and acid resistance at 3 pH levels (3.0, 4.0, and 5.0), it was determined that several bacterial isolates would possibly function well as indicators of *E. coli* O157:H7 destruction. The potential indicators were all slightly more resistant to heating and acid treatment than *E. coli* O157:H7. The greatest reduction for acid resistance occurred at pH 3.0 for *E. coli*

O157:H7 strains ranging from 2.4-6.1 log₁₀ CFU/ml. Indicator bacteria similarly were reduced approximately from 1.3-6.8 log₁₀ CFU/ml. *E. coli* O157:H7 strains had the greater reduction for heat treatment at 65°C, the range was 3.2-4.8 log₁₀ CFU/ml, while indicator bacteria similarly were reduced from 2.4-6.8 log₁₀ CFU/ml.

All tests were performed on stationary phase cells as well as in acid-acid adapted and non-acid acid adapted cells. The results of this study demonstrated that regardless of the physiological state of the cell, *E. coli* O157:H7 destruction was enhanced with increasing temperature and decreased pH. Testing indicated that several isolates from cattle hides would make good process control indicators since the indicator bacteria were determined to be similar or slightly more resistant than *E. coli* O157:H7 after assessed for heat- and acid resistance in this study.

Further testing of indicator bacteria in a commercial setting is necessary to determine usefulness for validation and verification of carcass intervention treatments. Use of effective indicator bacteria will significantly affect the beef industry, as many currently used carcass decontamination treatments have never been validated or verified to the satisfaction of regulatory agencies.

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