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The effect of freezing on the survival of *Escherichia coli* O157:H7 on beef trimmings

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Abstract

The ability of three *Escherichia coli* O157:H7 strains to survive on beef trimmings stored at −18°C over a 12 week period was determined. This was achieved by plating samples at weekly intervals on non-selective tryptic soy agar (TSA) and selective sorbitol McConkey agar (SMAC) to determine both total numbers and the degree of sub-lethal injury of this pathogen. Two freezing temperatures (−18 or −35°C) to simulate slow or rapid freezing respectively, and two inoculation levels (10^3 cfu g^{-1} or 10^4 cfu g^{-1}) were used. Further, the ability of an immunoassay (VIP) to detect this pathogen at the end of the frozen storage period was assessed. Counts on TSA remained constant, with a few exceptions, for all treatments while those on SMAC decreased significantly ($P \leqslant 0.05$) between 0.5 and 2 log cfu g^{-1} over the study period. The VIP test confirmed the findings of the plating experiments. These findings indicate the presence of varying degrees of sub-lethal injury among strains of this pathogen during frozen storage. The inability to eliminate *E. coli* O157:H7 and the presence of sub-lethally injured strains which are still infectious but undetectable on selective media make freezing an unreliable method to assure the safety of beef trimmings.

Keywords: Beef; *Escherichia coli* O157:H7; Freezing; Survival

1. Introduction

Escherichia coli O157:H7 is a foodborne bacterial pathogen of great public health concern due to the increasing number of outbreaks and the high mortality rate (Phillips, 1999) associated with this organism. Various food types have been implicated in *E. coli* O157:H7 outbreaks, including apple juice (Besser et al., 1993), vegetables (Ackers et al., 1998) and milk (Chapman, Wright & Higgins, 1993), but beef and beef products remain the most common vehicles for infection by this pathogen (Padhye & Doyle, 1992; Phillips, 1999). The infective dose of *E. coli* O157:H7 may be as low as 10 cells (Bolton, Crozier & Williamson, 1996) and infected individuals may shed the organism for up to 57 days, often resulting in person-to-person transfer (Williams, Hamilton, Wilson & Estock, 1997). For these reasons many countries have imposed strict regulations and controls on the presence of this pathogen in foods and food processing environments at both the national and international level.

Several methods to control contamination of beef carcasses have recently been developed and tested for efficacy against *E. coli* O157:H7. These methods, which include the use of antimicrobial washes (Cutter & Siragusa, 1994), steam vacuum (Dorsa, Cutter & Siragusa, 1997), organic acids (Dorsa, Siragusa, Cutter, Berry & Koohmaraie, 1997) and electric fields (Liu, Yousef & Chism, 1997), have various degrees of success in reducing or eliminating this pathogen. Since *E. coli* O157:H7 is a relatively recently emerged pathogen, the ability of traditional methods of preservation, such as freezing, to reduce contamination by this organism are also being examined (Sage & Ingham, 1998a). These investigations are important since such traditional technologies are often not subject to the regulatory problems associated with implementing newly developed techniques and may, therefore, be erroneously viewed by commercial companies as an easy way to assure the absence of this and other foodborne pathogens in their products.

Survival of *E. coli* O157:H7 during frozen storage and thawing has been investigated for a number of food products including ground beef (Sage & Ingham, 1998a), apple juice (Sage & Ingham, 1998b) and chicken (Conner & Hall, 1994). The survival of this pathogen in

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each of these frozen foods was highly variable and depended on the strain of *E. coli* O157:H7 inoculated onto the foods, as well as the freezing and thawing conditions. These findings clearly show that each frozen food system needs to be examined independently with respect to its ability to reduce or eliminate this pathogen and thus add a margin of safety to the product.

Escherichia coli O157:H7 may become sub-lethally injured by the various treatments, such as heating, freezing and the action of sanitisers, that they may be exposed to in foods (McCleery & Rowe, 1995; Stephens & Joynson, 1998; Taormina, Rocello, Clavero & Beuchat, 1998). Under such conditions, cells of this bacterium may be present on a product and able to cause disease but undetectable because they are sensitive to components of the selective agar used in their isolation (Clavero & Beuchat, 1995; Semancheck & Golden 1998). While some studies investigating survival of *E. coli* O157:H7 in foods have used non-selective agars (Conner & Hall, 1994) many others have used only selective agars (Sage & Ingham, 1998a,b) and may thus have underestimated survival of this bacterium in the product. In any study examining survival of this pathogen it is, therefore, important to quantify, if possible, the degree of sub-lethal injury as compared to actual death of bacterial cells in the product.

Beef trimmings destined for grinding are frequently transported frozen, particularly at an international level, and subsequently ground in a semi-thawed state on arrival at the processing plant. While the ability of *E. coli* O157:H7 to survive in ground beef has been examined (Sage & Ingham, 1998a), the ability of this pathogen to survive on beef trimmings pre-grinding has not been reported. Further, the degree of sub-lethal injury to cells stored in such products has not been determined. The present study was undertaken to determine the effect of simulated commercial freezing regimes on the survival and sub-lethal injury of *E. coli* O157:H7 on beef trimmings.

2. Materials and methods

2.1. Meat sample preparation

Fresh 90 CL (chemical lean) grade beef trimmings were obtained from a commercial meat plant. The trimmings were cut into smaller unequal sized pieces ranging from 100 to 300 g and portioned into 1 kg batches in plastic luncheon meat casings that were clipped at one end. Triplicate batches of meat were prepared for each combination of bacterial strain, inoculum level, freezing treatment and sampling period used in this study. In addition, triplicate batches were prepared as uninoculated controls for the above samples, as appropriate.

2.2. Bacterial cultures

Three strains of *E. coli* O157:H7 were used in this study: B6-914 gfp-91, E27 and CDC 2988. Strain B6-914 gfp-91 is a non-toxigenic laboratory marker strain genetically modified to express green fluorescent protein (Fratamico, Deng, Strobaugh & Palumbo, 1997); strain E27 is a toxigenic strain isolated from healthy dairy cattle by our laboratory (Buncic & Avery, 1997); and strain CDC 2988 is a toxigenic strain of human clinical origin obtained from Environmental Science and Research Ltd. (Christchurch, New Zealand). All strains were maintained on Protect Bacterial Preservers (Technical Service Consultants Ltd., Heywood, Lancashire, UK) at −85°C and resuscitated before use by three successive inoculations into tryptic soy broth (TSB) (Difco, Detroit, Michigan, USA) followed by incubation at 37°C for 24 h.

2.3. Experimental design

For each freezing treatment and sampling period, triplicate 1 kg batches of meat in plastic casings were inoculated with 10 ml of a TSB culture (18 h at 37°C) of one of the three strains diluted in 0.1% peptone/0.85% sodium chloride to give a final concentration of either 10^3 or 10^4 g^{-1}. These concentrations were selected as the lowest level of inoculum still detectable by the methods used in the present study and thus as close as possible to natural levels of contamination by *E. coli* O157:H7. The open end of the casing was tied, then the inoculated trimmings were massaged to distribute the inoculum throughout the meat. Inoculated batches were packed into cartons (18 per carton) and frozen at either −18 or −35°C to simulate slow or rapid freezing, respectively. After 24 hr all meat batches were transferred to −18°C and stored for up to 12 weeks (84 days). Based on a pilot study meat cartons were assumed to have reached a core temperature of between −17 and −22°C in this time with the −35°C stored samples cooling approximately 1.8 times faster than the −18°C samples (data not presented). Samples were selected at random and analysed microbiologically before freezing, immediately after freezing and at three-week (21 day) intervals during storage. Statistical significance was determined by analysis of variance using SigmaStat 2.0 (Jandel Scientific, California, USA).

2.4. Microbiological analysis

Before freezing, immediately after freezing and at three-week (21 day) intervals up to 12 weeks (84 days) frozen storage, composite 25 g samples were taken from three sites along the length of filled casings selected for analysis. With fresh batches samples were removed with sterile scalpel blades; with frozen batches samples were removed frozen by using a sterile hand saw to cut slices.

Fresh or frozen slices were placed in 225 ml 0.1% peptone/0.85% sodium chloride and stomached (Seward Stomacher 400, London, UK) for 2 min. Appropriate serial dilutions of the homogenate were plated, in duplicate, on both tryptic soy agar (TSA) (Difco, Michigan, USA), a non-selective medium, and sorbitol McConkey agar (SMAC) (Difco, Michigan, USA), a selective medium. This was done to determine the degree of sub-lethal injury of strains since the difference of counts on selective and non-selective agar represents a measure of this. Plates were incubated at 37°C for 24 h and results reported as $\log_{10}$ colony forming units (cfu) g^{-1}. To determine the numbers of *E. coli* O157:H7 on SMAC, typical non-sorbitol fermenting colonies were counted, with appropriate uninoculated samples acting as a negative control. On TSA all colonies were counted and a background reading from appropriate uninoculated controls was subtracted to give an estimate of *E. coli* O157:H7 numbers. The accuracy of these methods was checked by counting fluorescent colonies of strain B6-914 gfp-91 under a hand-held short-wave UV light (Merck, Darmstadt, Germany) and comparing these counts to the estimated counts of that strain.

At the end of the 12 week (84 day) storage period, an extra composite 10 g sample was taken from the final batches and stomached for 2 min in 90 ml of modified EC broth (Difco, Michigan, USA) with novobiocin. Stomacher bags were incubated at 37°C for 24 h before being tested for *E. coli* O157:H7 antigens using the VIP visual immunoprecipitate assay (Biocontrol Systems Inc., Washington, USA) with appropriate positive controls. Results were reported as positive or negative. This method is used routinely for screening of samples of beef trimmings taken in New Zealand for regulatory purposes in response to standards required for export markets. The mild enrichment protocol allows recovery of injured cells and confirms survival of this pathogen on frozen meat.

3. Results and discussion

The accuracy of the method for estimating *E. coli* O157:H7 counts on TSA and SMAC was acceptable since no significant differences between the corrected estimation for the fluorescent strain and the corresponding count under UV light were apparent. This use of a genetically marked bacterial strain highlights further potential for such strains for food survival studies in addition to those already reported (Fratamico et al., 1997). Further, at the lowest dilution (10^3 cfu g^{-1}) of *E. coli* O157:H7 inoculated they were present at levels at least 10 fold higher than the background bacterial counts in all cases, making them more likely to be isolated and thereby adding further confidence to the validity of the results.

Changes in the mean counts of the three strains of *E. coli* O157:H7 on TSA and SMAC for the two freezing treatments over the 12 week (84 days) period of this study are presented in Figs. 1–3. The microbiological methods used allowed detection of bacteria to counts

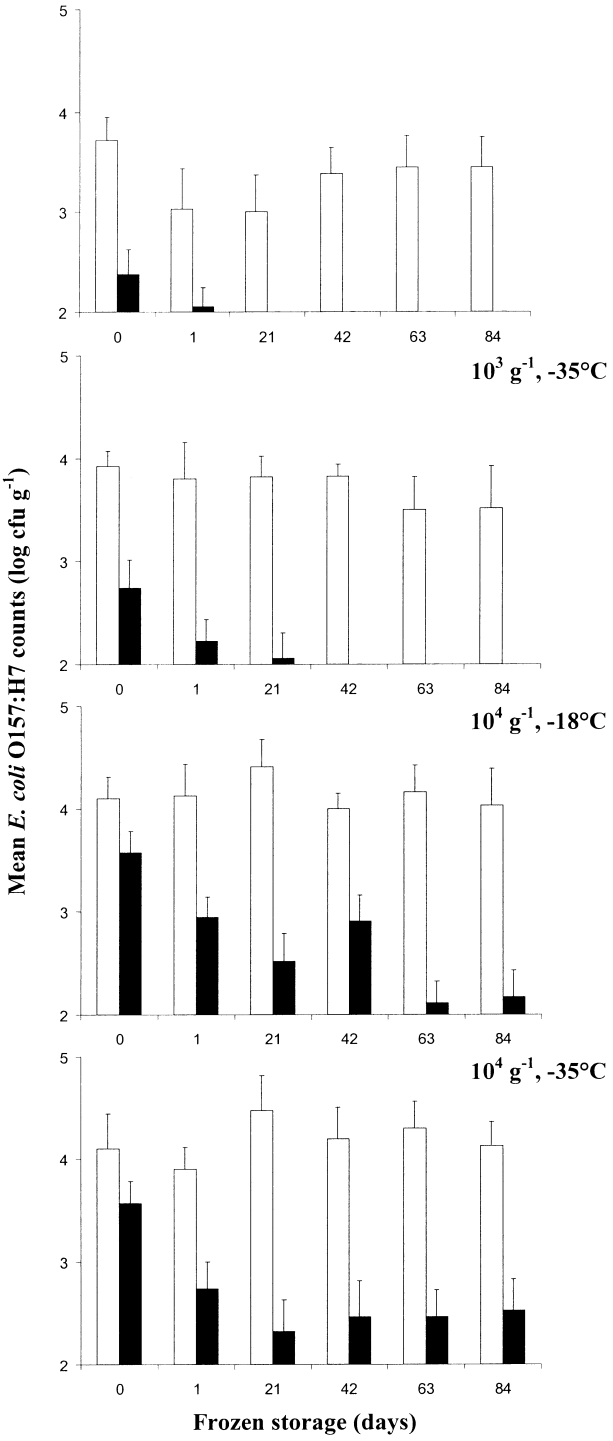

Fig. 1. Mean ($n=3$) *Escherichia coli* O157:H7 B6-914 gfp-91 counts and standard deviations (error bars) on TSA (light bars) and SMAC (dark bars) before and during frozen storage. Day 0 = before freezing. Limit of detection of method is 2 log cfu g^{-1}.

only as low as log 2 cfu g^{-1} and, therefore, this is the lowest level presented in the figures.

In all cases, before freezing initial counts on TSA were slightly higher than on SMAC. This difference is probably due to *E. coli* O157:H7 having a natural sensitivity to the highly selective components in SMAC. This feature was strain-dependent and most marked in the 10^3 g^{-1} dilutions of strains B6-914 gfp-91 and CDC 2988, which were the only examples of significant ($P \leqslant 0.05$) pre-freezing differences in counts between the two

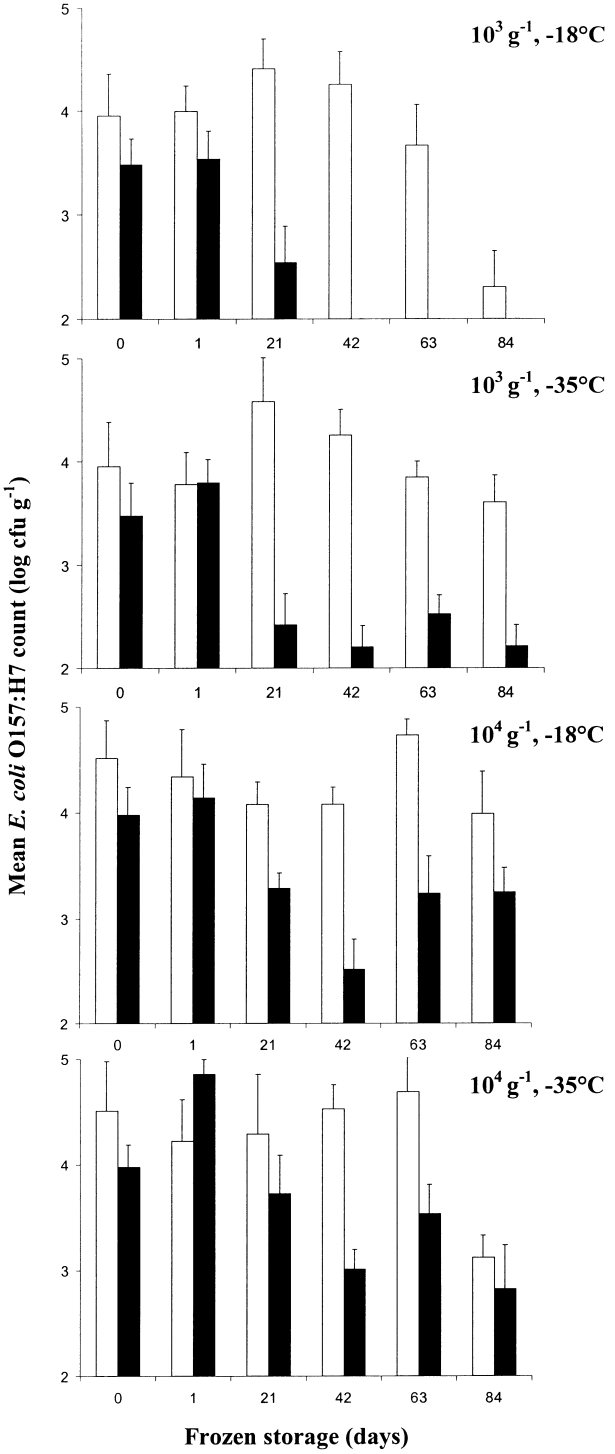

Fig. 2. Mean ($n = 3$) *Escherichia coli* O157:H7 E27 counts and standard deviations (error bars) on TSA (light bars) and SMAC (dark bars) before and during frozen storage. Day 0 = before freezing. Limit of detection of method is 2 log cfu g^{-1}.

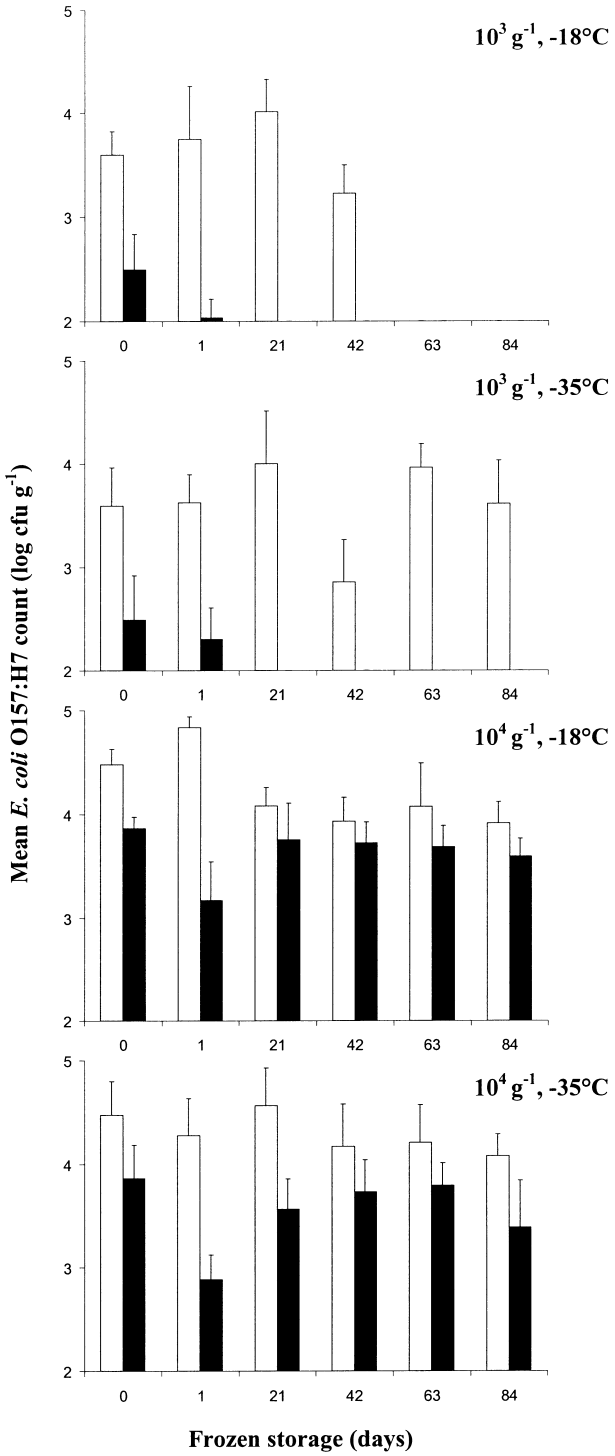

Fig. 3. Mean ($n = 3$) *Escherichia coli* O157:H7 CDC 2988 counts and standard deviations (error bars) on TSA (light bars) and SMAC (dark bars) before and during frozen storage. Day 0 = before freezing. Limit of detection of method is 2 log cfu g^{-1}.

media. Strain-dependent differences in stress response to components (such as bile salts) of SMAC have been reported in previous studies (Clavero & Beuchat, 1995; Stephens & Joynson, 1998) and, therefore, are not unexpected. Sensitivity to media components is an important consideration when detecting or counting this pathogen and may be exacerbated by sub-lethal injury of strains subjected to treatments such as freezing (Semancheck & Golden, 1998). In this study corrected *E. coli* O157:H7 counts on both TSA and SMAC were used to circumvent this.

Immediately after freezing and for the 12 week (84 days) storage period of the study, *E. coli* O157:H7 estimates on TSA for all strains, inoculation levels and freezing regimes, with two exceptions, decreased only slightly or not at all. Therefore, in most cases, freezing and frozen storage resulted in little or no death of inoculated *E. coli* O157:H7. However, when frozen slowly (−18°C freezing temperature) and initially present at the lower inoculation level (10^3 g^{-1}), strains E27 and CDC 2988 displayed a significant reduction ($P \leqslant 0.05$) to near or below the detection limit of the methods used in this study. Slow freezing of strains E27 and CDC 2988 therefore appeared to result in enhanced cell death after storage. Slower freezing is reported to result in greater injury to cells than rapid freezing (Sage & Ingham, 1998a) and thus this finding was not unexpected.

Counts of *E. coli* O157:H7 on SMAC for all strains, inoculation levels and freezing regimes, with two exceptions, decreased significantly ($P \leqslant 0.05$) and often dropped below the limit of detection of the method used, over the period of the study. The exceptions were for the 10^4 inoculum of strain CDC 2988 subjected to both the fast and the slow freezing regimes. These counts remained relatively constant over the entire storage period, although they fell slightly immediately after freezing. This apparent resistance to freeze-damage at higher inoculation levels may be due to a stationary-phase density dependent effect in this strain as has been reported for resistance to acid stress in *E. c*oli O157:H7 (Datta & Benjamin, 1999). Since TSA counts changed little over the course of the study, the reduction in numbers observed on SMAC is probably due to sub-lethal injury, but not death, among frozen *E. coli* O157:H7 strains (Clavero & Beuchat, 1995). The difference in counts on the two media, like the degree of sensitivity to the SMAC medium, varied from strain to strain, with the laboratory strain B6-914 gfp-91 being the most sensitive and the other two strains producing variable results, depending on levels of inoculation. The inconsistencies observed were not unexpected and are probably the result of high variability among counts as numbers approached the limit of detection. Overall trends were, however, clear and allow freezing to be assessed as an intervention strategy for *E. coli* O157:H7.

This study has shown that initial slow freezing and storage at −18°C for up to 12 weeks (84 days) of beef trimmings contaminated with *E. coli* O157:H7 may, for some strains, render low levels of contamination undetectable by currently used standard methods. This feature was, however, strain dependent and, therefore, freezing cannot be relied on to eliminate *E. coli* O157:H7 from product naturally contaminated by that organism. For most strains and freezing treatments, apparent sub-lethal injury rather than cell death occurred. This is different from findings previously reported for frozen beef patties where reductions of up to 2.50 log cfu g^{-1} were seen (Sage & Ingham, 1998a). This difference may be explained by the use of selective media alone to isolate cells of *E. coli* O157:H7 in this previous study (Sage & Ingham, 1998a) probably resulting in an overestimation of cell death. The importance of using non-selective media, in addition to traditionally used selective media, in bacterial survival studies is highlighted by this finding. Further, the potential health risks that may be encountered by not detecting living cells of this pathogen when isolated from frozen product straight onto selective media only are of concern.

In addition, the observed difference between the present and previous studies on ground beef may also be due to the fact that the characteristics of beef trimmings and ground beef are different. Specifically, beef trimmings may provide more protection during freezing since the fat portion of trimmings is distributed unevenly on the outside of the meat rather than evenly throughout, as is the case after grinding. In addition, beef trimmings have a much smaller surface to volume ratio than ground beef allowing greater exposure of the cells to the damaging effects of freezing.

At the end of 12 weeks storage, the VIP test indicated that *E. coli* O157:H7 antigens were present after 24 h enrichment in all inoculated samples except for the three samples inoculated with strain E27 at 10^3 cfu g^{-1} subjected to slow freezing (frozen at −18°C). These are also the only samples in which TSA counts were reduced to below the limits of detection of this study. Since regulatory samples are often tested for the presence of *E. coli* O157:H7 after enrichment by this immunological method, all samples with the single exception just mentioned would have been unacceptable. It should be noted, however, that levels of this pathogen inoculated onto meat for practical purposes of detection in this study are many times higher than would be expected for natural contamination. Furthermore, one triplicate inoculated sample was rendered safe, as assessed by the standard detection methods used, even at these high inoculation levels.

It can be concluded that while freezing may add a margin of safety to beef trimmings contaminated with *E. coli* O157:H7, the unpredictable and strain- dependent nature of cell destruction makes freezing an unreliable

method for product decontamination. However, since freezing promoted sub-lethal damage of cells, freezing could be expected to act synergistically with other pre-freezing intervention strategies and appropriate combinations of these treatments could present a reliable regime to assure beef is free of this pathogen. At the same time sub-lethal damage may create health risks of its own by allowing potentially pathogenic cells to go undetected on frozen product. For this reason, examination of other intervention strategies, such as antimicrobial washes, in combination with traditional preservation methods, such as freezing, should be researched further with respect to *E. coli* O157:H7.

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