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Factors effecting survival of Escherichia coli O157:H7 during freezing

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EXECUTIVE SUMMARY

Escherichia coli O157:H7 is the major human pathogen of current concern with respect to fresh meat. Freezing is widely used in the Australian meat industry to preserve manufacturing cuts during transport over long distances, particularly to Australia's major trading partners. Since freezing is known to damage and inactivate bacteria it has been suggested that this process may add an extra margin of safety to product with respect to *E. coli* O157:H7 as compared to unfrozen cuts. Results of various studies investigating the effect of freezing on the survival of *E. coli* O157:H7 on beef have been contradictory due to difficulty in obtaining controlled data. For this reason a previous MLA project (PRMS.049) developed a laboratory based method for obtaining controlled data on *E. coli* O157:H7 during freezing on beef. Using this method it was established that there was no significant reduction in numbers of this pathogen on beef during simulations of industry freezing profiles. In all cases the inoculum applied to the meat in PRMS.049 was from a 24hr culture grown in nutrient broth at 37^{o}C. *Escherichia coli* O157:H7 cells present on frozen boxed beef may be in varying physiological states due to exposure to different growth conditions and stresses, or to being in different phases of growth. These stresses, whether occurring before or together with freezing, may influence their ability to survive on meat. For this reason this study was undertaken to establish the effect of variations in growth conditions and stress on the ability of *E. coli* O157:H7 to survive freezing under controlled conditions established in PRMS.049. This report addresses the effect of six of these, namely water activity, growth temperature, different pH, growth phase of cultures, growth in liquid or on solid medium and freezing on fat or muscle.

Three *E. coli* O157:H7 strains were used in this study and enumerated separately, before and after freezing, on beef under a typical industry profile. Before being inoculated onto meat and frozen cells were either: grown at 37^{o}C and then exposed to three different water activities (0.96, 0.98 and 0.99) at 8oC for 14h; grown at three different temperature (15^{o}C, 25^{o}C and 37^{o}C) to late log phase; grown at 37^{o}C and then exposed to three different levels of pH (4.5, 5.5 and7) at 8^{o}C for 2h; grown at 37^{o}C to lag, mid-logarithmic and early stationary phase; grown at 37^{o}C on liquid or solid medium or grown at 37^{o}C and then frozen on beef muscle or fat only. For the water activity experiments slight reductions in numbers (0.12 to 0.23 log cfu g^{-1}) were

observed depending on the strain and water activity but these were not significantly different (P>0.05) from each other. For the temperature experiments reductions in numbers (0.08 to 0.60 log cfu g^{-1}) were observed. Reductions observed at 25^{o}C were significantly higher for all strains than those observed at the other two temperatures. In addition, strain EC119 displayed a significantly higher (P<0.05) reduction in numbers at 25^{o}C than the other two strains at the same temperature. For the pH experiments slight reductions in numbers (0.26 to 0.38 log cfu g^{-1}) were observed depending on the strain and pH but these were not significantly different (P>0.05) from each other. For the growth phase experiments slight reductions in numbers (0.13 to 0.20 log cfu g^{-1}) were observed depending on the strain and growth phase but these were not significantly different (P>0.05) from each other. For the substrate type experiments slight reductions in numbers (0.18 to 0.27 log cfu g^{-1}) were observed depending on the strain and substrate but these were not significantly different (P>0.05) from each other. For the fat/muscle experiments slight reductions in numbers (0.12 to 0.15 log cfu g-1) were observed depending on the strain and substrate but these were not significantly different (P>0.05) from each other.

It was established in this study that small non-significant reductions in numbers of all strains were apparent after freezing under the conditions of the study. The variations in water activity, two of the temperatures applied in this study, exposure to different pH, growth phase of cultures, growth in liquid or on solid medium and freezing on fat or muscle substrate appeared not to influence this reduction, but cells grown at 25^{o}C appeared more sensitive to freezing. The reduction in numbers of cells grown at 25^{o}C was, however, still relatively small and is not of practical significance. Based on this study and the previous one (PRMS .049) the only possible way examined for applying freezing as a control for this pathogen is to alter the freezing profile and extend the plateau phase.

It was concluded that the stresses and growth conditions applied in this study had very little influence on the ability of *E. coli* O157 to survive on meat under a typical industry freezing profile. While many of the stresses were applied sequentially with freezing, rather than simultaneously, the absence of almost any effect indicates that freezing under current industry conditions cannot be regarded as an intervention, even in the presence of other stresses.

1.0 INTRODUCTION

The toxigenic O157:H7 serotype of *Escherichia coli* is the major human pathogen of curren concern with respect to fresh meat safety worldwide and more specifically with regard to export of meat from Australia. Its detection in exported manufacturing cuts in other countries could result in a significant amount of product being condemned causing substantial economic loss to the producer and damaging market access for the meat industry as a whole.

Freezing is widely used in the Australian meat industry to preserve manufacturing cuts during transport over long distances, particularly to our major trading partners. Since freezing is known to damage and inactivate bacteria it has been suggested that this process may add an extra margin of safety to product as compared to unfrozen cuts [1]. A number of studies have investigated the effect of freezing on the survival of *E. coli* O157:H7 on beef trim and ground beef. Results of these studies have been contradictory with a decrease in numbers of cells on both selective (damaged cells) and non-selective (inactivated cells) media reported in some cases [2,3]. By contrast a decrease in cell numbers on selective but none on non-selective media was reported in other studies [4,5]. Furthermore, decreases observed were strain specific and often demonstrated a high degree of variability in bacterial counts between samples [6,7]. The exact freezing profiles used in these studies, particularly those on beef trim, were either undefined or demonstrated a poor relationship to actual industry practice. For these reasons a previous study entitled “An initial investigation into the effect of freezing on the survival of *Escherichia coli* O157:H7 in Australian boxed beef (PRMS.049)” was commissioned by MLA.

In the PRMS.049 project a highly controlled laboratory based method for simulating industry freezing practices and their effect on *E. coli* O157:H7 was developed and applied. It was established that the method was effective but that none of the typical industry freezing profiles used resulted in a significant reduction in numbers of this pathogen. An artificial profile which extended exposure of the pathogen to the

temperature range of between 0 and -2.5^{o}C did, however, result in a significant ($p<0.05$) decrease of ~1.2 log cfu g^{-1} in numbers of this pathogen [8].

In all cases the inoculum applied to the meat in PRMS.049 was from a 24hr culture grown in nutrient broth at 37^{o}C. It well established that the physiology of bacteria is influenced by the conditions under which they are grown or to stresses they are exposed to, and that this in turn influences their ability to survive other subsequent or simultaneous stresses applied to them [9,10]. *Escherichia coli* O157:H7 cells present on frozen boxed beef may be in varying physiological states due to exposure to different growth conditions and stresses, or to being in different phases of growth. These stresses, whether occurring before or together with freezing, may influence their ability to survive on meat. Exposure to low water activity at the surface of a carcass during chilling, followed by subsequent freezing, represents an example of such a potential effect. This project was undertaken to establish the effect of variations in six parameters on the ability of *E. coli* O157:H7 to survive freezing on beef under the controlled typical simulated conditions established in PRMS.049.

2.0 OBJECTIVES

The objectives of this study were to:

- Establish the effect of the following parameters on the survival of three Australian *E. coli* O157:H7 isolates on beef trim during freezing under simulated commercial profiles:
  - Water activity
  - Growth temperature
  - Exposure to different pH
  - Growth phase of cultures
  - Growth in liquid or on solid medium
  - Freezing on fat or muscle

- To re-assess the potential of freezing as an intervention and, if positive, suggest further research avenues to maximize benefit from this.

3.0 METHODS

3.1 Bacterial strains and maintenance

Three *E. coli* O157 strains were chosen for this study from the *E. coli* culture collection at Food Science Australia. The cultures were selected to represent different strains that may be encountered on Australian beef and/or go on to cause human disease. These cultures were designated:

- EC119 which is an *E. coli* O157:H7 strain isolated from a beef carcass in an Australian abattoir and which is known to have the stx1, stx2, eae, ehlyA virulence genes and is therefore potentially pathogenic.
- EC1820 which is an *E. coli* O157:H7 strain isolated from a human fatality and which is known to have the stx2, eae, ehlyA virulence genes.
- EC200 which is an *E. coli* O157:H- strain isolated from a beef carcass in an Australian abattoir and which is known to have the stx1, stx2, eae, ehlyA virulence genes and is therefore potentially pathogenic.

All cultures were revived from stocks stored at -80oC on Protect Bacterial Preservers (Technical Service Consultants) and checked for purity by growth at 37oC for 18h on Nutrient Agar (NB, Oxoid).

3.2 Culture preparation

Cultures for each of the different parameters under study were prepared described below. In each case cells grown at in NB at 37^{o}C and pH 7 for 24h were included as a reference control as these conditions were the same as those used in to grow cells in the previous project PRMS.049.

Water activity. For the water activity experiments cells were initially grown in 10ml of NB for 24h ± 1h at 37^{o}C. The 10ml cultures were pelleted by centrifugation (5min at 3000g) and washed three times in 0.85% NaCl before being resuspended in 10ml of NB adjusted to three water activities (0.96, 0.98 and 0.99) using NaCl. The values represented low (0.96) and high (0.98) water activities that may be encountered on beef carcasses during chilling as well as a typical water activity of NB (0.99). After resuspension cells were held at 8^{o}C for 14h to simulate chilling, before being inoculated onto meat as described below.

Growth temperature. For the growth temperature experiments cells were grown in 10ml of NB. Cultures were incubated at 15^{o}C, 25^{o}C or 37^{o}C. Cultures incubated at 37^{o}C were incubated for 24h and inoculated onto meat as described below. After growth the density of these cultures, as determined by absorbance at 550nm, was determined. Cultures incubated at 15^{o}C and 25^{o}C were monitored for absorbance at 550nm until they reached the equivalent of the cultures grown at 37^{o}C cultures at 24h. This was done to ensure all cells were in equivalent phases of growth. The cells grown at 15^{o}C and 25^{o}C were then inoculated onto meat as described below.

Exposure to different pH. For the pH experiments cells were initially grown in 10ml of NB for 24h at 37^{o}C. The 10ml cultures were pelleted by centrifugation (5min at 3000g) and washed three times in 0.85% NaCl before being resuspended in 10ml of NB adjusted to three pH's (4.5, 5.5, or 7) using lactic acid. Samples were incubated in 8℃ waterbath for 2h to simulate chilling, befor e being inoculated onto meat as described below.

Growth phase of cultures. For the growth phase experiments cells were initially grown in 1L NB for at 37^{o}C. Cells in lag phase, mid-logarithmic phase and early stationary phase (as established from growth curve determinations) were pelleted by centrifugation (5min at 3000g) and resuspended at the same density as cultures described above (as determined by optical density at 550nm) in 10ml NB. Cells were then innoculted onto meat as described below.

Growth in liquid or on solid medium. For the liquid growth medium experiments cells were grown in 10 ml of NB for 24h at 37^{o}C. For the solid growth medium experiments 200µl of cell suspension from a 24h NB culture were spread plate on a Nutrient Agar (NA, Oxoid) plate (10ml) and incubated for 24h at 37^{o}C. The entire lawn of growth was resuspended in 10ml NB and adjusted to the same cell density (550nm) as the liquid culture. Both liquid and resuspended solid cultures were inoculated onto meat as described below.

Freezing on fat or muscle. For the fat and muscle experiments cells were grown in 10ml of NB for 24h at 37^{o}C and inoculated onto meat or fat as described below.

3.3 Inoculation and freezing procedure

For all experiments except fat and meat comparisons small plugs (1g) of fresh beef trim were prepared using a sterile cork-borer and scalpel, and taking care to provide a cross-section of both fat and muscle. For the meat and fat experiments small plugs (1g) of fresh beef trim fat or lean muscle were prepared separately in the same way as for mixed plugs. The plugs were immersed for 15s in the inoculum and then allowed to dry on a paper towel for 5min. The plugs were placed in 1ml cryogenic vials (Nalgene, USA) with the lids removed and the tops subsequently sealed using Parafilm “M” (American National Can, USA). The cryogenic vials were placed in a Cryochamber (Cryologic, Australia) connected to a CL-3300 Temperature Controller (Cryologic), TL-13 Temperature Logger (Cryologic) and computer running CryoGenesis V5 (Cryologic) software. The Cryochamber was placed in a -80oC freezer and a typical industry median freezing profile established in PRMS.049 (Fig. 1) and lasting 40h was used in this study and run to completion. This profile previously resulted in no reduction in numbers of any of the three *E. coli* O157:H7 strains used in this study.

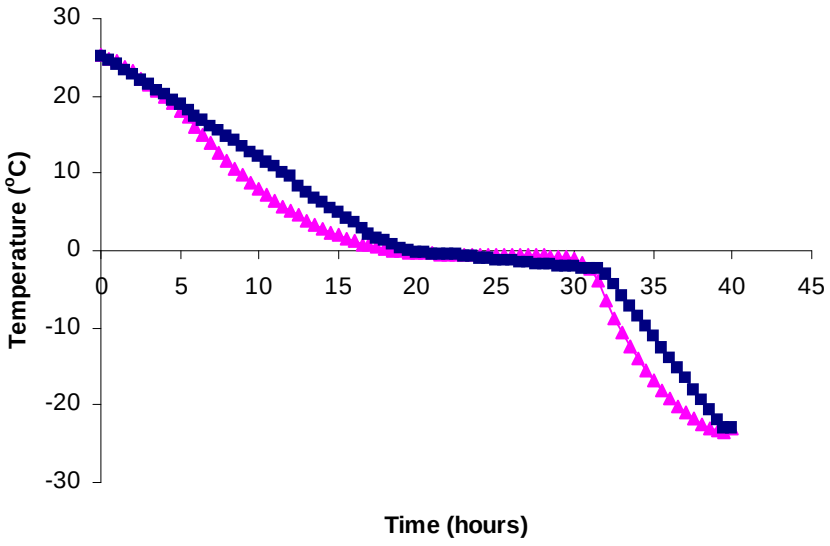

Fig 1. Profile of industry recorded (▲) and associated simulated (■) median (40h) freezing regime starting at 25°C.

3.4 Enumeration of *E. coli* O157:H7

Immediately before and after completion of the freezing protocol separate meat plugs were analysed for *E. coli* O157 numbers. This was achieved by placing the plugs in 9ml of Buffered Peptone Water (Oxoid) and vortexing them vigorously for 2min.

Appropriate dilutions of this original sample were then made and plated on NA plates which were incubated at 37^{o}C for 24 hours and counted. In all cases uninoculated controls were included in the analysis and these as well as pilot experiment data indicated that all bacteria counted on the plates were
the inoculated *E. coli* O157. Experiments for each bacterial strain under each freezing protocol were replicated five times to ensure low variability.

3.5 Statistical analysis

Counts were determined per gram of meat before and after freezing and mean log reductions were calculated from these counts. The results were analysed for significance using ANOVA and Tukey's multiple-range test in Minitab® 14 (Minitab Inc., USA).

4.0 RESULTS

4.1 Effect of water activity on freezing survival

The mean differences between counts before and after freezing for the three *E. coli* O157:H7 strains and water activities used in this study are presented in Fig. 2. Reductions ranged from 0.12 to 0.23 log cfu g^{-1} depending on the strain and water activity but these differences in number were not significant (P>0.05) for any of the combinations investigated.

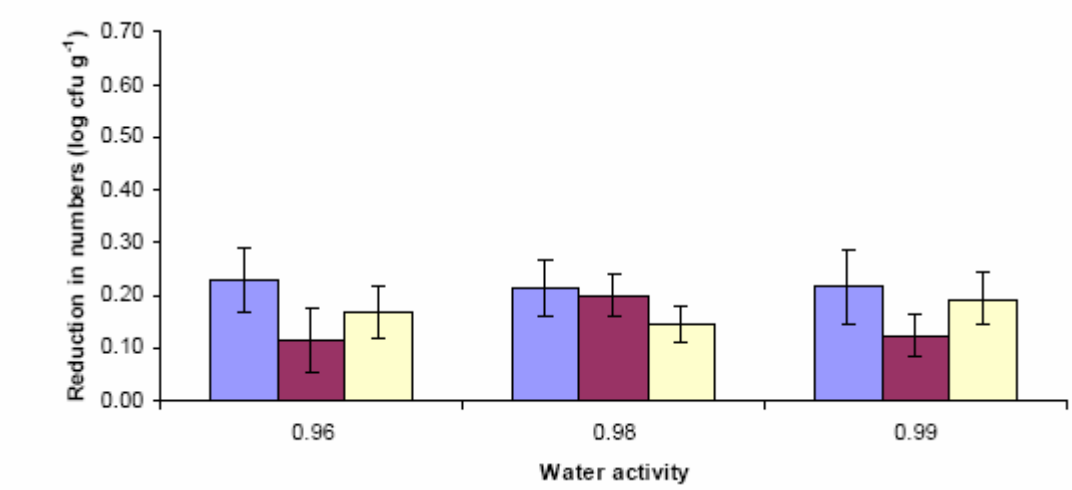

Fig 2. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) on beef during freezing after pre-treatment at 8^{o}C at three different water activities.

4.2 Effect of growth temperature on freezing survival

The mean differences between counts before and after freezing for the three *E. coli* O157:H7 strains and growth temperatures used in this study are presented in Fig. 3. Reductions ranged from 0.08 to 0.60 log cfu g^{-1} depending on the strain and temperature. No significant differences (P>0.05) in reduction in numbers were apparent between any of the combinations of strain and growth temperature at 15^{o}C and 37^{o}C. The reduction in numbers at 25^{o}C for all *E. coli* O157:H7 was, however, significantly higher (P<0.05) than for the same strains at both 15^{o}C and 37^{o}C. Furthermore, strain EC119 displayed a significantly higher (P<0.05) reduction in numbers at 25^{o}C than the other two strains at the same temperature.

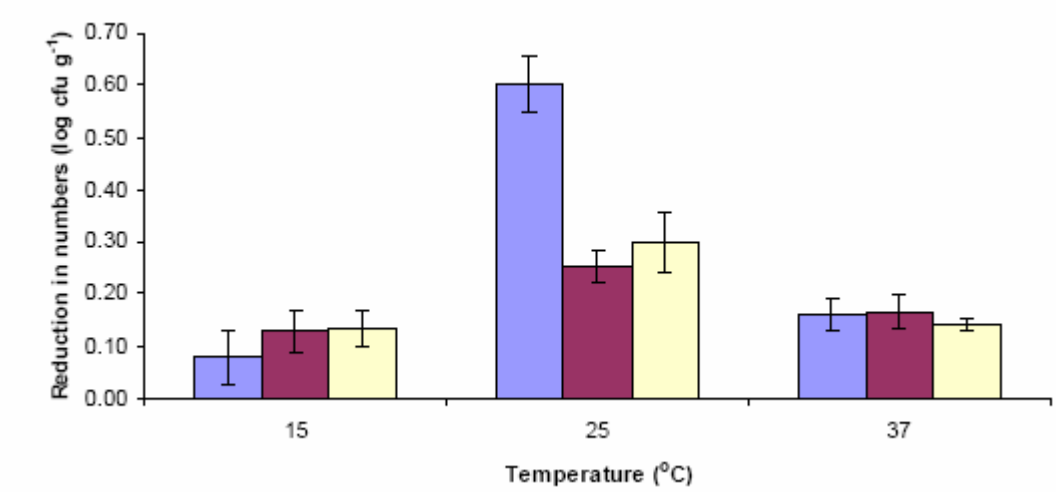

Fig 3. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) on beef during freezing after growth at three different temperatures.

4.3 Effect of exposure to different pH on freezing survival

The mean differences between counts before and after freezing for the three *E. coli* O157:H7 strains and pH used in this study are presented in Fig. 4. Reductions ranged from 0.26 to 0.38 log cfu g^{-1} depending on the strain and pH but these differences in number were not significant (P>0.05) for any of the combinations investigated.

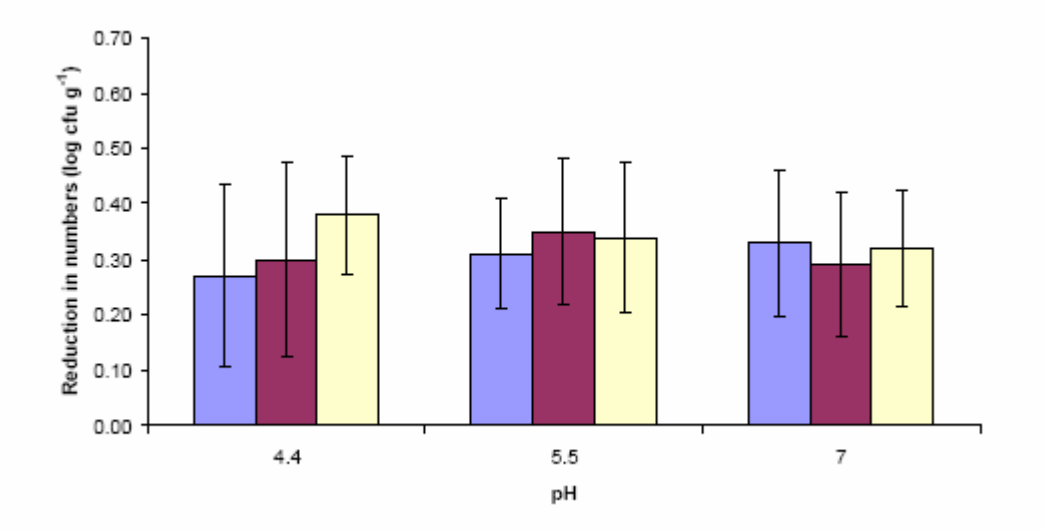

Fig 4. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) on beef during freezing after exposure to three different pH at 8°C.

4.4 Effect of growth in liquid or on solid medium on freezing survival

The mean differences between counts before and after freezing after growth on liquid or on solid medium for the three *E. coli* O157:H7 strains used in this study are presented in Fig. 5. Reductions ranged from 0.18 to 0.27 log cfu g^{-1} depending on the strain and substrate but these differences in number were not significant (P>0.05) for any of the combinations investigated.

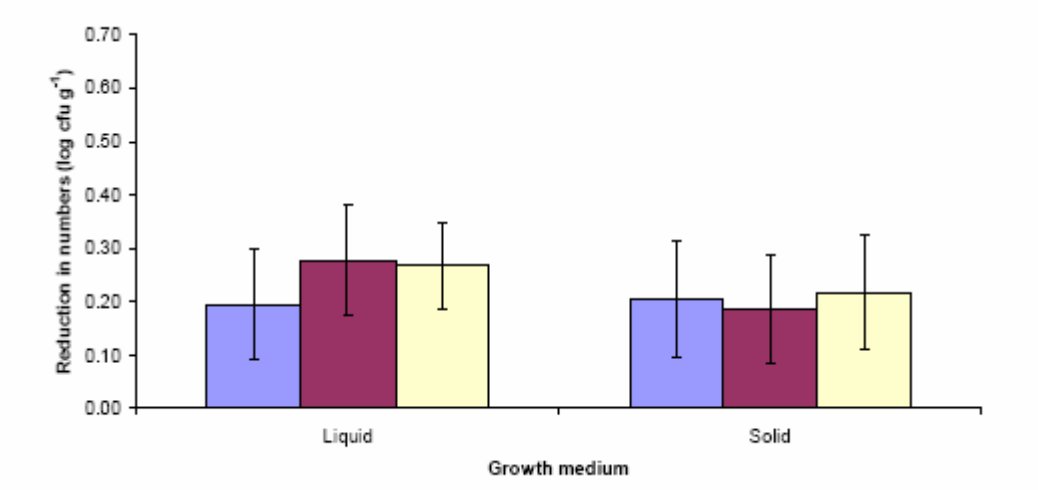

Fig 5. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) after growth in liquid or on solid medium.

4.5 Effect of growth phase of culture on freezing survival

The mean differences between counts before and after freezing for the three *E. coli* O157:H7 strains and growth phases are presented in Fig.6. Reductions ranged from

0.13 to 0.20 log cfu g^{-1} depending on the strain and growth phase but these differences in number were not significant (P>0.05) for any of the combinations investigated.

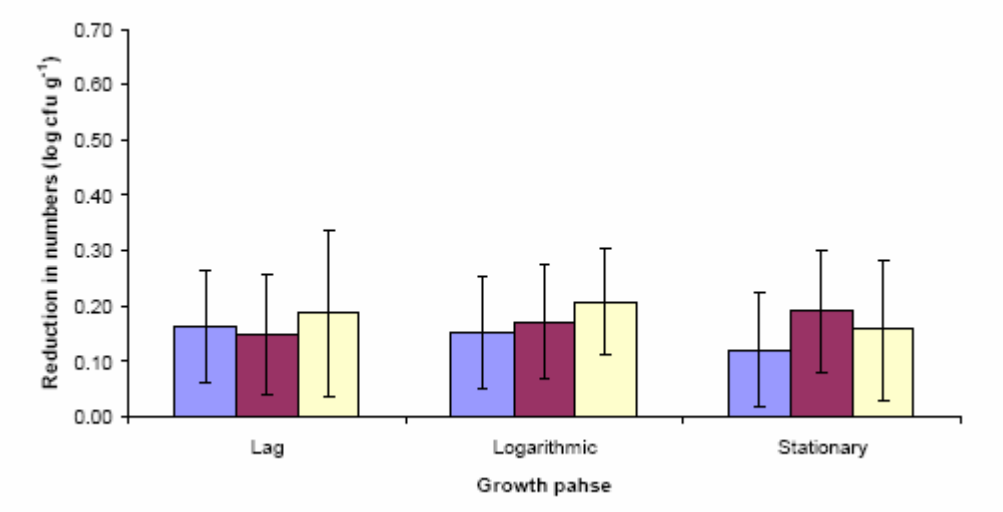

Fig 6. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) on beef during freezing at different phases of the growth cycle.

4.6 Effect of freezing on fat or muscle on survival

The mean differences between counts before and after freezing on either beef fat or muscle for the three *E. coli* O157:H7 strains used in this study are presented in Fig. 7. Reductions ranged from 0.12 to 0.15 log cfu g^{-1} depending on the strain and substrate but these differences in number were not significant (P>0.05) for any of the combinations investigated.

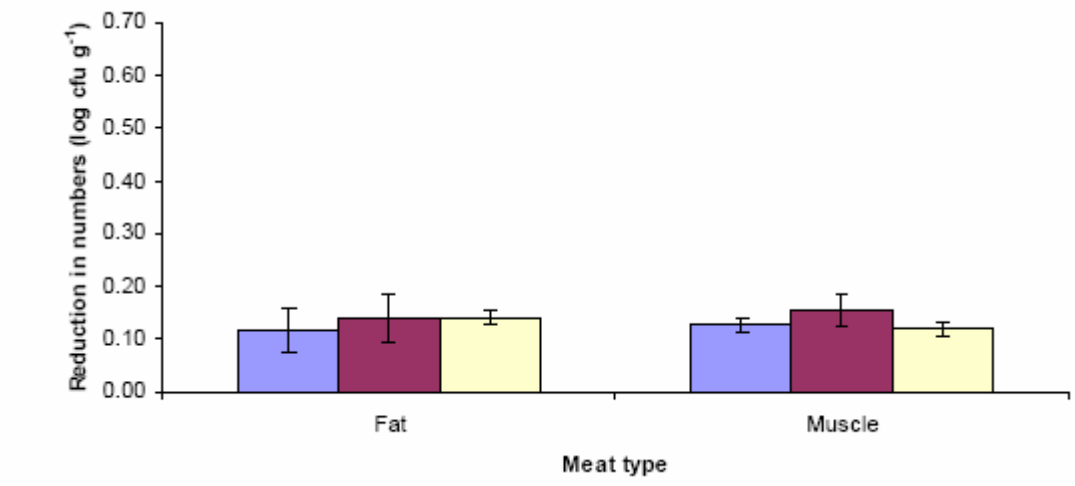

Fig 7. Reduction in numbers of three strains of *E. coli* O157: H7 (EC119, EC200 and EC 1820 from left to right) after freezing on beef fat or muscle.

5.0 DISCUSSION

In this study numerical data on *E. coli* O157:H7 was presented as means of reductions in numbers, rather than separate before and after counts, for ease of presentation and comparison. Although not a specific aim of this study, it should however be noted that the differences between before and after counts for all strains and variables, except growth at 25^{o}C, were not statistically significant (P>0.05). These findings are consistent with the results of the previous study PRMS .049, in which no significant differences for before and after counts were observed for the freezing profile used. This data points to the high degree of consistency between results over time and between operators using this controlled method.

The absence of an effect of exposure to lower water activity at low temperature on the sensitivity of *E. coli* O157:H7 cells are of note. It has been established that osmotic stress may damage cells [11] and therefore assumed that the dual stresses of lower water activity and freezing may act synergistically. Anecdotal evidence has also suggested that drying of carcasses before freezing may result in an enhanced reduction in *E. coli* O157:H7. Two reasons may be forwarded for the absence of an effect observed in the present study. The first is that the highly controlled nature of the experiments has eliminated other variables and influences present on carcasses under industrial conditions and that there is no effect of lower water activity per se. The method used in the current study, however, does not allow for the simultaneous application of osmotic and freezing stress on meat which may occur in industry. A method to simultaneously apply both stresses would be useful to confirm this hypothesis. The second is that under lower water activity bacterial cells are protected through the uptake of compatible solutes [11]. This in turn also protects them from the freezing process, which in part also exerts its effect through osmotic stress [1]. In either case, unravelling this phenomenon is unlikely to result in a reduction in *E. coli* O157:H7 of industrial value.

Only one of the variables investigated in the current study, namely growth of strains at 25^{o}C, produced a significantly different result from those obtained for 24hr cultures grown in nutrient broth at 37^{o}C. The reasons for this phenomenon are not clear. It might be expected that if lower growth temperatures influenced the survival of *E. coli*

O157:H7 during freezing then cells grown at 15^{o}C should also display increased sensitivity. It is well established, however, that cells respond to different environmental temperatures in various ways. It might be possible, for example, that compatible solutes which exert a protective effect were taken up at 15^{o}C and not 25^{o}C, thereby neutralising the sensitivity induced at 25^{o}C [11]. Regardless of its cause the reduction observed was not large (0.60 log cfu g^{-1} or lower) and therefore is unlikely to be of practical significance as it stands. Furthermore, it is not obvious at what temperatures cells under industrial conditions have grown and a combination of temperatures may be expected.

The absence of an effect of exposure to different pH on the sensitivity of *E. coli* O157:H7 cells to freezing indicate that the use of acid sprays (as applied in countries other than Australia) is unlikely to act in synergy with freezing to kill this pathogen. *E. coli* is known to be able to be sensitive to, but also to resist, acid stress under a range of conditions [12]. Anecdotal evidence has also suggested that *E. coli* O157:H7 may also be more resistant to acid stress than other *E. coli*. In the current study all strains were of this serotype and it may be that they all had high acid tolerance. Regardless of this possibility, the presence of some the absence of synergism in some strains negates the usefulness of a combined pH/freezing approach to controlling this pathogen.

Growth on solid or in liquid medium, as well as phase of growth, has been shown to influence the ability of bacteria, including *E. coli*, to survive stress [13, 14]. This effect has been attributed to the fact that a wide array of genes are up or down regulated when bacteria grow on surfaces as opposed to in liquid, or at different phases of growth. In the current study none of the parameters tested appeared to influence survival during subsequent freezing. This was unexpected and points toward the fact that freezing may not be as severe a stress as others, such as chilling, where stationary phase cell, for example, tend to be more resistant to the stress. The results of this part of the study also indicate that small variations in test methodology will not strongly influence the results and that the system used is a robust one.

The absence of an effect of freezing on fat as opposed to muscle on the sensitivity of *E. coli* O157:H7 cells were an interesting and somewhat unexpected result. It has

been suggested fat is better able to protect cells relative to the protection offered by lean muscle, but under the controlled conditions in this study this appears not to be the case. It may be that under more aggressive freezing regimes (which are not applied in industry) an effect is observable but this is of academic rather than practical interest.

In general, the absence of any influence of growth parameters on freezing survival of *E. coli* O157:H7 was a disappointing one. The test system, however, is robust and results should be taken as representative. Based on this study and the previous one (PRMS .049) the only possible way examined for applying freezing as a control for this pathogen is to alter the freezing profile and extend the plateau phase. The practical and quality consequences of such an approach are, however, not known at this point in time.

6.0 CONCLUSIONS

The stresses and growth conditions applied in this study had very little influence on the ability of *E. coli* O157 to survive on meat under a typical industry freezing profile. While many of the stresses were applied sequentially with freezing, rather than simultaneously, the absence of almost any effect indicates that freezing under current industry conditions cannot be regarded as an intervention, even in the presence of other stresses.

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