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Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in Food matrices.

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Abstract

E. coli O157:H7 or *E. coli* O26 which were antibiotic sensitive (AS); laboratory created antibiotic resistant mutants (AR); or naturally multi-antibiotic resistant (MAR), were inoculated into laboratory media, yoghurt or orange juice and their growth/survival monitored during enrichment at 37°C or storage at 4°C. The strains were also inoculated into minced beef and their thermal inactivation (D values) examined at 55°C, with and without a prior heat shock at 48°C. The growth kinetics (lag phases, growth rates) of the verocytotoxigenic *E. coli* (VTEC) incubated over 24 h at 37°C in laboratory media, were similar regardless of the presence or absence of antibiotic resistance. In yoghurt and orange juice, *E. coli* O157:H7 (MAR) died off significantly faster ($P < 0.05$), than any of other VTEC strains examined. *E. coli* O157:H7 (MAR) was also found to be significantly more heat sensitive ($P < 0.05$), than the other VTEC strains tested. The reasons for the observed differences in survival of the different VTEC strains and the link between antibiotic resistance and survival in VTEC organisms are discussed.

Introduction

Over the last 60 years there has been a huge increase in antibiotic usage (Levy, 1998) and the discovery of new antibiotics has decreased, with only one entirely new antibiotic (Zyvox) having been discovered in the last 40 years (Hall, 2004). This increase in antibiotic usage to control bacterial infection has allowed antibiotic resistant strains of bacteria to develop and proliferate. The direct clinical implications of the development and dissemination of antibiotic resistant genes has been a significantly reduced ability to effectively control an increasing range of human bacterial diseases (Levy, 1998; Nicolaou *et al.*, 2001).

An implication of antibiotic resistant bacteria, is that acquisition of antibiotic resistance may influence the behaviour of such organisms during typical food processing conditions. Reports in the literature suggest that antibiotic resistant bacteria, may display different growth kinetics in laboratory media and may display different resistance to stresses, such as acid and heat. Despite this, there is a lack of studies on the relationship between antibiotic resistance and food-related stresses (Bacon *et al.*, 2003). In 1978, Park first reported that a number of antibiotic resistant mutant strains exhibited significantly slower growth rates, than their parent strains. Similarly, Blackburn and Davies (1994) reported slower growth rates among antibiotic resistant strains of *Escherichia coli* O157:H7, compared to antibiotic sensitive strains. VTEC strains like *E. coli* O157:H7 have a high acid tolerance and have been associated with outbreaks in low pH foods such as juice and dairy products (Martin *et al.*, 1986; Besser *et al.*, 1993). Recent reports suggest a possible link between the acquisition of antibiotic resistance and survival of *E. coli* O157:H7 at a low pH (Mc Gee, 2003). While acid resistant pathogens such as VTEC are usually eliminated from dairy and juice products by pasteurisation, (re) contamination or inadequate pasteurisation (in the case of

fruit juices) is often a cause of food borne illness (Clark *et al.*, 1997; Anderson *et al.*, 2001). This suggests that research into factors, which may have the ability to alter typical VTEC survival patterns is warranted. Reports in the literature also suggest a possible link between the acquisition of antibiotic resistance and pathogen thermal resistance (Walsh *et al.*, 2001; Walsh *et al.*, 2005). This is of concern when dealing with pathogens, which have a low infectious dose such as *E. coli* O157:H7, particularly in the processing of ready-to-eat foods, such as fermented meat. A 5D treatment process is recommended for ready-to-eat foods, which would support the growth/survival of *E. coli* O157:H7. Any factor, which could impact on thermal resistance of VTEC and effect the design of thermal treatments process and safety margins, needs to be investigated.

To-date, antibiotic resistance in VTEC have received little research attention, possibly because VTEC strains have been reported slower to acquire resistance than generic *E. coli* strains (Mizan *et al.*, 2002; Sanchez *et al.*, 2002; Bettelheim *et al.*, 2003). However, more recently, multi-resistant strains of VTEC have been isolated from foods (Gallard *et al.*, 2001; Schroeder *et al.*, 2002(a); Schroeder *et al.*, 2002(b); Fitzgerald *et al.*, 2003; Schroeder *et al.*, 2003), suggesting increasing proliferation of antibiotic resistance among VTEC.

The objective of this study is to investigate how *E. coli* O157:H7 or *E. coli* O26 which were antibiotic sensitive; laboratory created antibiotic resistant mutants; or a naturally multi-antibiotic resistant strain, behaved in relation to 1) growth kinetics in laboratory media at 37°C 2) during storage of yoghurt and orange juice at 4°C or 3) thermal resistance in VTEC isolates at 55°C and to assess whether the acquisition of antibiotic resistance altered behaviour of the pathogens under the above conditions.

Materials and methods

Bacterial strains

E. coli O157:H7 (ATCC 43895) were obtained from the National Collection of Typed Cultures (NCTC), PHLS Central Public Health Laboratory, London. An additional natural multi-resistant strain (resistant to 10 antibiotics) of *E. coli* O157:H7 (MAR) was isolated from Irish minced beef (Cagney *et al.*, 2004) and *E. coli* O26 (M328) of human origin was obtained from PHLS, Cherry Orchard Hospital, Dublin, Ireland. Recommended control strains *E. coli* NCIMB 12210 (ATCC 25922) and *Staphylococcus aureus* NCIMB 12702 (ATCC 25923) were obtained from (NCIMB) The National Collection of Industrial and Marine Bacteria. The antibiotic resistant profiles for all of these strains was obtained in a study by Walsh *et al.* (2005) and are shown in Table 1. All strains of *E. coli* were stored on cryoprotect beads (Technical Consultant Services Ltd., Heywood, Lancashire, UK) at -20°C . Antibiotic resistant profiles were determined using the Bauer- Kirby Disc Diffusion Method (Bauer *et al.*, 1966), as described in the (NCCLS) National Committee for Clinical Laboratory Standards Performance Standards for Antimicrobial Disks (Anon., 2004). All VTEC strains studied were known to contain *vt1*, *vt2*, *eaeA* and *hlyA*, with the exception of *E. coli* O157:H7 (MAR) which was known to contain *vt1*, *eaeA* and *hlyA*, but no *vt2* (Cagney *et al.*, 2004).

Preparation of antibiotic resistant mutant (AR) from wild- type isolates

Antibiotic susceptible (AS) *E. coli* O157:H7 (ATCC 43895) and *E. coli* O26 (M328) were treated by the method of Blackburn and Davies (1994) to obtain mutants which were chromosomally resistant, to $50\ \mu\text{g ml}^{-1}$ nalidixic acid and $1000\ \mu\text{g ml}^{-1}$ streptomycin sulphate. The antibiotic resistant mutant of *E. coli* O157:H7 (ATCC 43895) was labelled *E. coli* O157:H7 (AR) and the

antibiotic resistant mutant from *E. coli* O26 (M328) was labelled as *E. coli* O26 (AR). Antibiotic status and stability was confirmed by growing these mutants on nutrient agar plates containing 50 µg ml⁻¹ nalidixic acid and 1000 µg ml⁻¹ streptomycin sulphate. All strains of *E. coli* were stored on cryoprotect beads (Technical Consultant Services Ltd., Heywood, Lancashire, UK) at -20°C.

Preparation of inoculum

Protect beads coated with one of the 5 VTEC serovars, *E. coli* O157:H7 (AS), *E. coli* O157:H7 (AR), *E. coli* O157:H7 (MAR), *E. coli* O26 (AS) or *E. coli* O26 (AR) were incubated in 30 ml volumes of Brain Heart Infusion Broth (BHI) (Oxoid) at 37°C for 24 h to produce stationary phase cultures. A 1.0 ml aliquot from each culture was transferred to 30 ml Brain Heart Infusion Broth (BHI) (Oxoid) and further incubated at 37°C for 18 h. The resultant cells were centrifuged (4500 g for 10 min at 4°C), and resuspended in 9 ml fresh Maximum Recovery Diluent (MRD) (Oxoid).

Growth in laboratory medium

Cell suspensions of each of the 5 VTEC strains, produced as described above, were inoculated into 300 ml volumes of Brain Heart Infusion (BHI, Oxoid) to give a final concentration of approximately 10² cells ml⁻¹ and incubated at 37°C for up to 24 h. Aliquots of 0.1 ml were removed from each culture every 15 min for the first hour and then every hour for 24 hours and plated out on Mc Conkey No.3 (Oxoid), in duplicate. The plates were then incubated at 37°C for 24 h and examined to provide estimates of VTEC numbers. This experiment was repeated on three separate occasions.

Growth / survival in yoghurt and orange juice

Cartons of natural yoghurt (100 g ~pH 4.2) and of fresh orange juice (1 l containing no preservatives, ~pH 4.4) were obtained from retail outlets in the Dublin area and their contents dispensed in 100 ml volumes, in 150 ml sterile containers (Sterilin). Each container was chilled to $4^{\circ}\text{C} \pm 0.5$ and inoculated with one of the five VTEC cell suspensions to a final concentration of 10^8 cells ml^{-1} . Aliquots (5 ml) were immediately withdrawn from each container and from uninoculated control samples (to provide T_0 samples) and the containers were held at $4^{\circ}\text{C} \pm 0.5$, for 25 days (yoghurt) or 35 days (orange juice). During storage, 5 ml samples were removed every 2 days up to day 14 and then every 3 days until day 35. At each sample occasion, the pH values of the inoculated samples, and uninoculated control samples were recorded. Numbers of VTEC in samples of yoghurt or orange juice were determined by a direct count, which involved plating 0.1 ml aliquots onto cefixime tellurite sorbitol Mc Conkey (CT-SMAC) (*E. coli* 0157) or cefixime tellurite-rhamnose Mc Conkey (CT-RMAC) (*E. coli* 026) (Catarama *et al.*, 2003) and incubating for 24 h at 37°C . A recovery count, was also performed which involved plating 0.1 ml aliquots of each sample onto Trytone Soya Agar (TSA, Oxoid), incubating at 25°C for 2 h, overlaying with (8-10 ml) of CT-SMAC / CT-RMAC, and then incubating at 37°C for a further 22 h, as previously described by Duffy *et al.* (1999). The differences between direct and recovery counts was used to estimate the numbers of sub-lethally injured cells present in these samples. This experiment was repeated on three separate occasions.

Thermal resistance in mince-meat

Beef trimmings (70% visible lean), obtained from an abattoir in the Dublin area, were minced (Crypto Ltd., London), dispensed in 100 g amounts, blast frozen at -30°C for 2 h in a blast freezer (Woods M3C₃, Avon Refrig. Co. Ltd. U.K.) and stored frozen at -20°C . Each batch of

mince beef samples was prepared from a single box of beef trimmings, and was confirmed as VTEC negative using the standard IMS cultural method (ISO 16654) before use in the experiment. Prior to use, each batch of mince samples was defrosted at 4°C overnight. Each of the above (5) VTEC cell suspensions were added to 10 g (± 0.5 g) volumes of mince-meat (n=3) and mixed with a sterile fork to give final bacterial concentrations of 10^6 - 10^7 cfu g⁻¹. The inoculated meat samples were then aseptically placed in bags (3 x 5 , 0.002 mm poly bags, Cole-Palmer Instruments Co., U.S.A.) and vacuum packed (Vac-Star S- 220, Verpackungsmaschinen AC, Switzerland). The study was repeated on two subsequent occasions, deriving fresh batches of mince-meat from separate boxes of trimmings.

Heat shock (HS) study

Vacuum packs of inoculated mince (n=3) were randomly assigned to one of two treatments. [a] “Heat shock (HS) treatment”. These packs were completely submerged in a waterbath equilibrated to 48°C ($\pm 1^\circ\text{C}$). Mince core temperature was monitored using thermocouples connected to a continuous temperature recording system (Ellab, Norfolk, UK) and placed in the centre of an uninoculated mince sample in a silicone resealed pack. When the centre of the control mince sample had reached the target temperature of 48°C (approximately 1 min), the inoculated packs were held at 48°C for a further 30 min, removed and cooled to below 5°C in an alcohol ice-bath within 2 min.

[b] “Non-heat shock (NHS) treatment”. Packs were held at 3°C ($\pm 1^\circ\text{C}$) for 30 min, HS samples were heat shocked. HS and NHS packs were stored in a chill room ($3 \pm 1^\circ\text{C}$) for approximately 10 min, and treated as described below.

Heat treatment

HS and NHS packs were immersed in a waterbath equilibrated to 55°C ($\pm 0.5^\circ\text{C}$). The internal temperatures of immersed samples were monitored as described above. The waterbath was stirred to ensure that the internal temperature of the mince samples in the packs reached 55°C within 1.5 min. Sample packs were removed at that time (t_0) and every 5 minutes for the next 50 min. Immediately after removal, each pack was placed in an alcohol ice-bath, cooled to below 5°C within 2 min, and held in a cold room ($3^\circ\text{C} \pm 1^\circ\text{C}$) for approximately 30 min, before microbiological analysis.

Microbiological examination of samples

Cooled samples were transferred to stomacher bags fitted with integral filters (Seward Ltd., London) and stomach for 2 min with 90 ml Maximum Recovery Diluent (MRD, Oxoid). The resultant filtrate was serially-diluted in MRD and examined by the direct and recovery methods described above. Six random colonies from each of the 3 replicate experiments, were confirmed using latex agglutination kits, Wellcolex (Merseyside, U.K.) for *E. coli* 0157:H7 and Denka Seiken (Denmark) for *E. coli* 026.

Antibiotic resistance profile

The antibiotic resistance profiles of each of the 5 strains recovered after heat treatment (including HS and NHS), were examined. This included colonies recovered in the final stages of heat treatment, after receiving heat challenge and possible heat shock and colonies which had received no heat treatment at all. This was conducted for the 3 replicate experiments which were carried out. Single colonies were taken from CT-SMAC/CT-RMAC plates and their antibiotic resistance

profiles determined by the method of Bauer *et al.* (1966). The profiles between the heat treated colonies were compared with the 5 corresponding non-heat treated colonies (results not shown). Thermal inactivation of the VTEC strains and antibiotic resistance profiling, was carried out independently on three separate occasions.

Statistical analysis

1. Growth in laboratory medium

The relationship between the count (in log units) and time (hours) for the growth phase, for each strain was examined, using linear regression analysis (Genstat 5, Rothamsted Experimental Station). The growth rates (slopes) obtained for each VTEC strain were compared using the t-test. The length of the lag phase was determined by substituting the initial inoculum values into the regression equation and solving as described by Duffy *et al.* (1994).

The regression equation $Y = MX + C$

was rearranged to $X = \frac{Y - C}{M}$

Where, X = time (predicted lag phase value, h), Y = population density (initial inoculum) [\log_{10} (cfu ml⁻¹)], C = intercept and M = slope.

2. Growth / survival in yoghurt and orange juice

The relationship between the count (in log units) and time (days) for each strain/medium was examined using regression analysis (Genstat 5, Rothamsted Experimental Station). Linear and quadratic equations were fitted to the data and the most appropriate model selected in each case. Goodness of fit values were calculated and rates of decline were compared using the t-test.

3. Thermal resistance in mince-meat

Linear regression analysis was used to relate the number (in log units) of bacteria of each strain undergoing each procedure [heat shock/non-heat shock], after each heating period (min) (Genstat 5, Rothamsted Experimental Station). D-values were calculated by determining the negative reciprocal of the slopes for the fitted line. The calculated slopes were then compared using a t-test, to assess whether there were differences in rates of decline for each strain.

Calculation of injury

The total population of cells that displayed injury was calculated as follows:

$$\text{Injury} = \text{TSA-SMAC counts} - \text{SMAC counts}$$

Results

1. Growth in laboratory medium

The growth rates of the examined strains of VTEC in laboratory medium are presented in Table 2. *E. coli* O157:H7 and O26 strains (AS, AR and MAR) were found to have similar growth rates (\log_{10} 0.74 to 0.77 cfu ml⁻¹). All the VTEC strains examined also had lag phases of similar duration, ranging between 0.83 to 1.30 h (Table 2).

1.1. Enumeration of acid and heat injured VTEC

The recovery count technique was used for samples likely to contain cells injured by stress (acid and heat) and was found to yield a higher VTEC count, than the direct count procedure. These higher counts obtained by recovery, suggest a significant level of injury in the stressed cells. The level of injury was similar for *E. coli* O157:H7 and *E. coli* O26 in yoghurt (\log_{10} 7.05 to 7.62 cfu

ml⁻¹) and juice (log₁₀ 7.14 to 7.83 cfu ml⁻¹). The level of injury was similar for the (AS) and (AR) strains of *E. coli* O157:H7 (log₁₀ 7.21 to 7.23 cfu ml⁻¹) and *E. coli* O26 (log₁₀ 6.71 to 7.18 cfu ml⁻¹) in meat. However *E. coli* O157 (MAR) which was resistant to 11 antibiotics, showed less injury (log₁₀ 5.99 to 6.34 cfu ml⁻¹), than other VTEC strains tested. Since injury is often only a temporary state, the recovery technique was considered to have yielded the most realistic results and so in this paper the results reported are from this technique only.

2. Survival in yoghurt and orange juice

The survival of the VTEC strains during storage over 25 days in yoghurt and 35 days in juice at 4°C, is shown in Figure 2 and 3. The pH of the yoghurt samples ranged from 4.1- 4.3 (± 0.1) during the study, while the pH of juice samples ranged from 4.2- 4.5 (± 0.1). VTEC were found to survive longer in juice (approximately 30 days), than in yoghurt (approximately 18 days). There was no significant difference between the rates of survival of (AS) and (AR) strains of either serovar (O157:H7/O26), regardless of medium (yoghurt/juice). *E. coli* O157 (MAR), however died off significantly faster (P<0.05), than all the other VTEC stains, in both yoghurt and juice.

3. Thermal Resistance in mince-meat

Thermal resistance (D-values) at 55°C for all VTEC strains (with or without a prior heat shock) is presented in Table 4. It is clear from the goodness of fit values (r²) in Table 4, that the rate of decline of bacteria with time is well explained by a negative linear relationship for all strains, with r² values ranging from 0.86 to 0.99. All heat shocked (48°C for 30 min) strains had similar D-values, to their non-heat shocked counterparts, with the exception of one strain *E. coli* O26 (AR), which was found to be significantly more heat resistant (11.14 min) than its non-heat

shocked strain (8.64 min) ($P < 0.05$). The D-values for both serovars and for (AS) and (AR) strains were greatly similar for *E. coli* O157:H7 (11.70 to 13.15 min) and *E. coli* O26 (9.73 to 12.19 min). However a significantly lower D-value was recorded for *E. coli* O26 (AR) (8.64 min). The multi-resistant strain of *E. coli* O157:H7 (MAR) was very thermal sensitive and had a recorded D-value of 1.71 min. The VTEC strains which were heat challenged and tested for a change in their antibiotic resistance profile following heat treatment, were found to have no difference in their profile from that of strains prior to heat challenging.

Discussion

This study examined the growth or survival of *E. coli* O157:H7 or *E. coli* O26, which were antibiotic sensitive (AS); laboratory created antibiotic resistant mutants (AR); or naturally multi-antibiotic resistant (MAR) in laboratory media, yoghurt or orange juice and minced beef.

This is one of the first studies to compare directly the growth kinetics and survival of *E. coli* O157:H7 and *E. coli* O26 during food enrichment and to compare their survival under typical processing conditions. No significant difference in growth kinetics; lag phase or growth rate (Table 3), was observed between the two serovars. Equally, the survival of these two serovars in low pH food and their thermal tolerance at 55°C in meat, was comparable. This indicates that where a process (based on low pH or heat) is validated to ensure absence of *E. coli* O157:H7, the same conditions should also be effective against *E. coli* O26. A considerable difference between the direct (selective) counts and recovery counts was observed, when VTEC strains were recovered from foods under stressed condition (acid and heat). This confirms that there is a significant level of injury in cells recovered from such foods, which have received sub-lethal processing treatments or from inhibitory environments (Juneja *et al.*, 1997; Duffy *et al.*, 1999;

Walsh *et al.*, 2001). It also validates the need for the use of such recovery methods on processed foods, to avoid an underestimate of the bacterial population.

The antibiotic resistance profiles for VTEC strains before and directly after a food processing stress (sub-lethal heating) were examined in this study and it was concluded that the process induced no change in the antibiotic resistance. This is in agreement with Walsh *et al.* (2005), who found no change in antibiotic resistance profiles before and after thermal treatment on chicken meat. These studies are however at variance with other published studies carried out in broth (Batish *et al.*, 1991; Walsh *et al.*, 2000), which (in the majority of cases) found that sub-lethal heat treatment rendered bacterial cells more susceptible to antibiotics. The difference in the conclusions from these various studies is likely to be related to the experimental methodologies employed, with heat treatment conducted in broth in previous studies and in mince-meat in the present study. It is well recognised that in meat, bacteria receive a less severe heat treatment (at the same temperature) than in broth, due to the protective nature of fat and protein in the meat. Further research work is needed to establish the effect, that sub-lethal heating of liquid foods such as milk and juice etc., has on antibiotic resistance profiles. This should be carried out not only on pathogenic bacteria, but also on the background micro-flora, which may for example survive pasteurisation, but remain a vector for transfer of antibiotic resistance in the food and subsequently to the consumer.

Laboratory-developed antibiotic resistance (AR) strains created by the method of Blackburn and Davies (1994), are commonly used in microbiology as “marked” strains, to facilitate ready detection of such pathogens from other phenotypically similar strains or species in experimental protocols (Dombroski *et al.*, 1999; Daly *et al.*, 2002; Prendergast *et al.*, 2004). In this study, the

impact of laboratory acquired antibiotic resistance to nalidixic acid and streptomycin on the growth and survival of *E. coli* O157:H7 (AR) and *E. coli* O26 (AR) was examined. This was done to facilitate a direct comparison of survival of antibiotic sensitive parent strains (AS) and laboratory acquired antibiotic resistant strains (AR). This also enabled some conclusions to be made about whether these laboratory developed “marked” strains are good indicators of how the wild-type organism would behave. The presence of (AR) did not effect the growth kinetics (lag phases, growth rates) of the VTEC strains, over a 24 h period at 37°C in BHI. This is in contrast to a study by Blackburn and Davies (1994), who found that some laboratory acquired antibiotic (nalidixic acid and streptomycin) resistance *E. coli* (AR) strains, exhibited slightly slower growth than their parent strains (AS). The survival of VTEC (AR) strains in orange juice and yoghurt and their D-values at 55°C were not significantly different to their parent strains. Although, it has been reported by McGee (2003) that acid sensitivity occurred in 1 of 3 *E. coli* O157:H7 strains, which had laboratory acquired nalidixic acid and streptomycin resistance. Studies in the literature on the effect of laboratory acquired nalidixic acid and streptomycin (AR) strains, on D values of *Listeria* and *Salmonella* (Walsh *et al.*, 2001; Walsh *et al.*, 2005) are in agreement with this study. It can be concluded from this study that laboratory acquired antibiotic resistance (AR) did not impact on the behaviour of VTEC under the conditions examined. This makes them very suitable as marked strains for use in experimental trials or process validations. The results suggest that when antibiotic resistance is induced as a result of chromosomal mutation as in the case of these laboratory developed strains, it does not appear to inflict any obvious fitness cost to the bacterium.

Studies were also conducted on the survival of a multi-antibiotic resistant *E. coli* O157:H7 strain

recovered (from a minced-meat sample), as part of a previous surveillance study conducted by this laboratory (Cagney *et al.*, 2004). This particular strain was resistant to 11 antibiotics and recent reports in the literature indicate that multi-antibiotic strains of VTEC are now being detected in other surveillance studies (Gallard *et al.*, 2001; Schroeder *et al.*, 2002(a); Schroeder *et al.*, 2002(b); Schroeder *et al.*, 2003). These studies include the isolation of multi-resistant strains of *E. coli* O157:H7 resistant to 9 or more antibiotics (Fitzgerald *et al.*, 2003). This investigation aimed to conduct an initial snap shot study, to see if this isolated strain with multi-antibiotic resistance behaved any differently to other VTEC strains. Studies showed that the growth kinetics; lag phases and growth rates (Table 3) of the *E. coli* O157 (MAR) over 24 h at 37°C in laboratory media (BHI) were similar to all other strains tested. However, when subjected to food stresses (acid and heat) this particular *E. coli* O157:H7 (MAR) isolate, was found to act very differently to the (AS) and (AR) VTEC strains tested. In yoghurt and orange juice, the (MAR) strain died off significantly faster ($P < 0.05$), than the other strains tested. Thermal inactivation studies showed the (MAR) strain to be significantly more heat sensitive (D_{55} value 1.71 min) than all other VTEC strains examined in this study, or indeed in the wider literature. D-values for *E. coli* O157:H7, inactivated in mince-meat at 55°C, reportedly range from 11.13 to 139.2 min in the literature (Juneja *et al.*, 1997; Clavero *et al.*, 1998; Byrne *et al.*, 2002; Huang *et al.*, 2003). It is not known if this particular (MAR) strain is an atypical clone with extreme sensitivity, or if its poor survival is a phenomenon related to its antibiotic resistance, as only one natural multi-resistant strain was available for examination during this study. Interstrain difference may have played a role and it is well recognised that there are wide interstrain differences in survival among *E. coli* strains (Duffy *et al.*, 1999; Kimmit *et al.*, 2000; Durso *et al.*, 2004). However, if the poor survival of the (MAR) strain in this study, is truly linked to antibiotic resistance, then several stress response mechanisms used by the pathogen in dealing with food processing stresses

may play a role. *RpoS* genes required for survival of *E. coli* at low pH (Waterman *et al.*, 1996) may be involved. *RpoS* is a highly mutable gene (Siegele *et al.*, 1992) and mutations to this gene due to the acquisition of antibiotic resistance, may have resulted in acid and heat sensitivity in *E. coli* O157:H7 (MAR) (Zambrano *et al.* 1993; Rowe *et al.*, 1999). Another stress response gene *slp*, which is involved in the uptake of nutrients or medium constituents (Seone *et al.*, 1995) is also reported to be down regulated by the MAR system (Price *et al.*, 2000) and may be another possible link to the extreme acid and heat sensitivity observed in this *E. coli* O157:H7 (MAR).

Regardless of the cause, this study shows that particular clones of *E. coli* O157:H7 can behave relatively differently when subjected to food-borne stresses. Thus, when designing and validating food safety process margins, validation trials should always be conducted using control strains which are recognised as having a tolerance to stress which is at the higher end of the spectrum for the pathogenic group. With the growing reports of multi-antibiotic resistant strains it is clear that further research is now needed on a larger numbers of (MAR) VTEC strains. This is needed to identify both if (MAR) resistance is generally plasmid or chromosomal located and to assess whether pathogen survival is effected by the presence of multi-antibiotic resistance genes.

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Table 1:
Antibiotic resistance profiles of *E. coli* O157 (AS, AR and MAR) and *E. coli* O26 (AS, AR)

Antibiotics	Disk Content (µg)	Equivalent MIC (µg/ml)	<i>E. coli</i> O157		<i>E. coli</i> O26		<i>E. coli</i> O157 MAR
			AS	AR	AS	AR	
Ampicillin	10	32	-	-	-	-	R
Kanamycin	30	25	-	-	-	-	R
Cefixime	5	4	-	-	-	-	-
Cefachlor	30	32	-	-	-	-	-
Streptomycin	10	-	-	R	-	R	R
Trimethoprim	5	16	-	-	-	-	R
Nalidixic Acid	30	32	-	R	-	R	R
Rifampicin	5	4	R	R	R	R	R
Sulphonamides	300	350	-	-	-	-	R
Chloramphenicol	30	32	-	-	-	-	R
Tetracycline	30	16	-	-	-	-	R
Minocycline	30	16	-	-	-	-	R
Ciprofloxacin	5	4	-	-	-	-	-
Doxycycline	30	16	-	-	-	-	R
Moxalactam	30	64	-	-	-	-	-
Norfloxacin	10	16	-	-	-	-	-

* R- indicates resistance to a particular antibiotic.

† Equivalent Minimum Inhibitory Concentration (MIC) breakpoints are µg/ml of antibiotic to which the bacteria is resistant to, as per the NCCLS Standard (Anon., 1997). The NCCLS Standard does not list a MIC for Streptomycin.

‡ AS: antibiotic susceptible, AR: antibiotic resistant, MAR: multiple antibiotic resistant

Table 2: Growth Rate \log_{10} (cfu/ml/h) and lag phase (h) for VTEC strains (*E. coli* O157 AS, AR, MAR and *E. coli* O26 AS, AR) in BHI at 37°C.

Serovar	a	Growth Rate (b)	Residual d.f.	Lag phase (h)
<i>E. coli</i> O157 AS	1.344 \pm 0.149	0.7667 \pm 0.0257	6	0.85 \pm 0.1097
<i>E. coli</i> O157 AR	1.032 \pm 0.136	0.7425 \pm 0.0220	5	1.30 \pm 0.2633
<i>E. coli</i> O157 MAR	1.264 \pm 0.121	0.7694 \pm 0.0195	5	0.95 \pm 0.2258
<i>E. coli</i> O26 AS	1.094 \pm 0.192	0.7576 \pm 0.0261	4	1.05 \pm 0.3419
<i>E. coli</i> O26 AR	1.380 \pm 0.062	0.7435 \pm 0.0113	7	0.83 \pm 0.1617

* The linear regression is $y = a + bt$ where $y = \log$ count, $a = \text{constant}$, $b = \text{linear regression coefficient}$ and $t = \text{time (hours)}$.

† AS: antibiotic susceptible, AR: antibiotic resistant, MAR: multiple antibiotic resistant

Table 3: The relationship between antibiotic resistance and survival in low pH foods (yoghurt and orange juice) for VTEC strains (*E. coli* O157 AS, AR, MAR and *E. coli* O26 AS, AR), during storage at 4°C.

Serovar	Antibiotic Status	Goodness of fit (%)	a	b	c	Residual d.f
Yoghurt						
<i>E. coli</i> O157	AS	81	8.35 \pm 0.90	-0.15 \pm 0.22	-0.009 \pm 0.011 ^e	7
<i>E. coli</i> O157	AR	97	8.17 \pm 0.37	-0.10 \pm 0.08	-0.011 \pm 0.003 ^e	8
<i>E. coli</i> O157	MAR	98	8.68 \pm 0.34	-0.69 \pm 0.10	0.012 \pm 0.006 ^d	5
<i>E. coli</i> O26	AS	93	8.16 \pm 0.51	0.01 \pm 0.11	-0.016 \pm 0.005 ^e	9
<i>E. coli</i> O26	AR	97	7.94 \pm 0.34	0.17 \pm 0.08	-0.028 \pm 0.007 ^e	7
Juice						
<i>E. coli</i> O157	AS	95	7.69 \pm 0.39	0.20 \pm 0.06	-0.011 \pm 0.002 ^e	9
<i>E. coli</i> O157	AR	94	7.54 \pm 0.41	0.22 \pm 0.06	-0.012 \pm 0.002 ^e	10
<i>E. coli</i> O157	MAR	96	8.17 \pm 0.34	-0.04 \pm 0.05	-0.006 \pm 0.001 ^d	10
<i>E. coli</i> O26	AS	94	7.82 \pm 0.41	0.18 \pm 0.06	-0.011 \pm 0.002 ^e	10
<i>E. coli</i> O26	AR	92	7.68 \pm 0.57	0.23 \pm 0.86	-0.013 \pm 0.002 ^e	9

* The quadratic regression is $y = a + bt + ct^2$ where $y = \log$ count, $a = \text{constant}$, $b = \text{linear regression coefficient}$, $c = \text{quadratic regression coefficient}$ and $t = \text{time (days)}$.

† Regression coefficients with different superscript are significantly different ($p < 0.05$).

‡ AS: antibiotic susceptible, AR: antibiotic resistant, MAR: multiple antibiotic resistant

Table 4: D₅₅ values (min) for VTEC strains (*E. coli* O157 AS, AR, MAR and *E. coli* O26 AS, AR) heated in mince meat, with or without a prior heat shock at 48°C.

Strain	Antibiotic Status	Treatment	Selective Recovery					Non-selective Recovery				
			D-value	Slope	S.E.	D.F.	r ²	D-value	Slope	S.E.	D.F.	r ²
<i>E. coli</i> O157:H7	AS	NHS	10.04	0.10 ^a	0.01	6	0.94	11.70	0.09 ^c	0.01	8	0.97
<i>E. coli</i> O157:H7	AS	HS	13.42	0.08 ^b	0.00	9	0.98	13.15	0.08 ^c	0.01	8	0.97
<i>E. coli</i> O157:H7	AR	NHS	9.50	0.11 ^a	0.01	7	0.98	11.36	0.09 ^c	0.01	9	0.97
<i>E. coli</i> O157:H7	AR	HS	9.93	0.10 ^a	0.01	8	0.92	11.90	0.08 ^c	0.01	9	0.95
<i>E. coli</i> O157:H7	MAR	NHS	0.90	1.11 ^c	0.08	3	0.98	1.71	0.59 ^f	0.10	6	0.86
<i>E. coli</i> O157:H7	MAR	HS	1.11	0.90 ^c	0.05	6	0.98	1.51	0.66 ^f	0.06	8	0.94
<i>E. coli</i> O26	AS	NHS	8.18	0.12 ^a	0.01	7	0.94	9.73	0.10 ^{eg}	0.01	7	0.98
<i>E. coli</i> O26	AS	HS	10.61	0.09 ^a	0.01	7	0.95	12.19	0.08 ^c	0.01	7	0.96
<i>E. coli</i> O26	AR	NHS	7.20	0.14 ^d	0.01	6	0.99	8.64	0.12 ^g	0.01	8	0.99
<i>E. coli</i> O26	AR	HS	8.70	0.12 ^a	0.01	9	0.89	11.14	0.90 ^c	0.01	7	0.90

* D-values in the same column with no letter in common are significantly different (P<0.05).

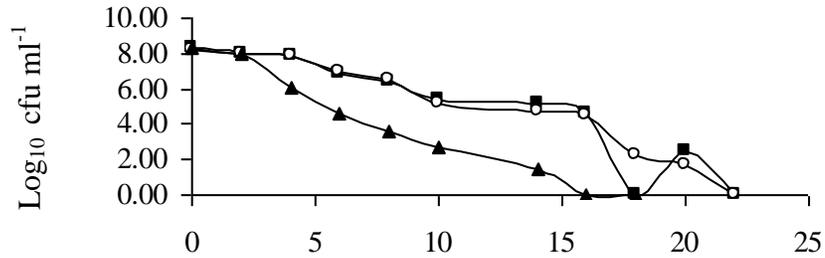
† HS: Heat Shocked at 48°C for 30 min, NHS: Non-Heat Shock

‡ AS: antibiotic susceptible, AR: antibiotic resistant, MAR: multiple antibiotic resistant

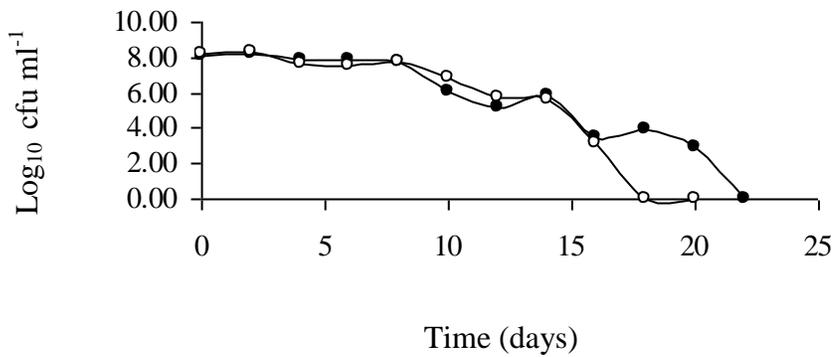
Figure 1. Survival of VTEC strains (*E. coli* O157 AS, AR, MAR and *E. coli* O26 AS, AR) in yoghurt at 4°C.

Figure 2. Survival of VTEC strains (*E. coli* O157 AS, AR, MAR and *E. coli* O26 AS, AR) in orange juice at 4°C.

A. *E.coli* O157

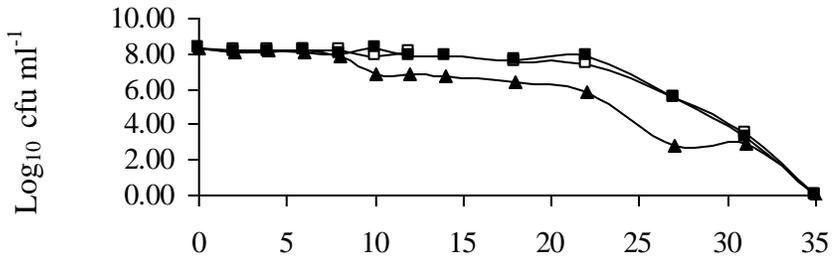


B. *E.coli* O26

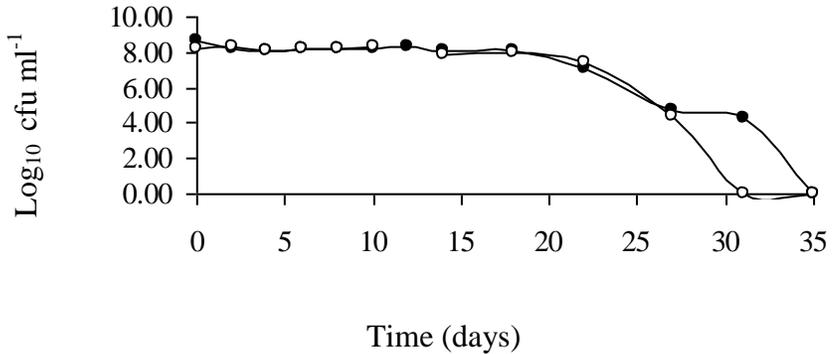


**E.coli* O157 AS (□), *E.coli* O157 AR (■), *E.coli* O157 MAR (▲), *E.coli* O26 AS (●), *E.coli* O26 AR (○)

A. *E.coli* O157



B. *E.coli* O26



**E.coli* O157 AS (□), *E.coli* O157 AR (■), *E.coli* O157 MAR (▲), *E.coli* O26 AS (●), *E.coli* O26 AR (○)