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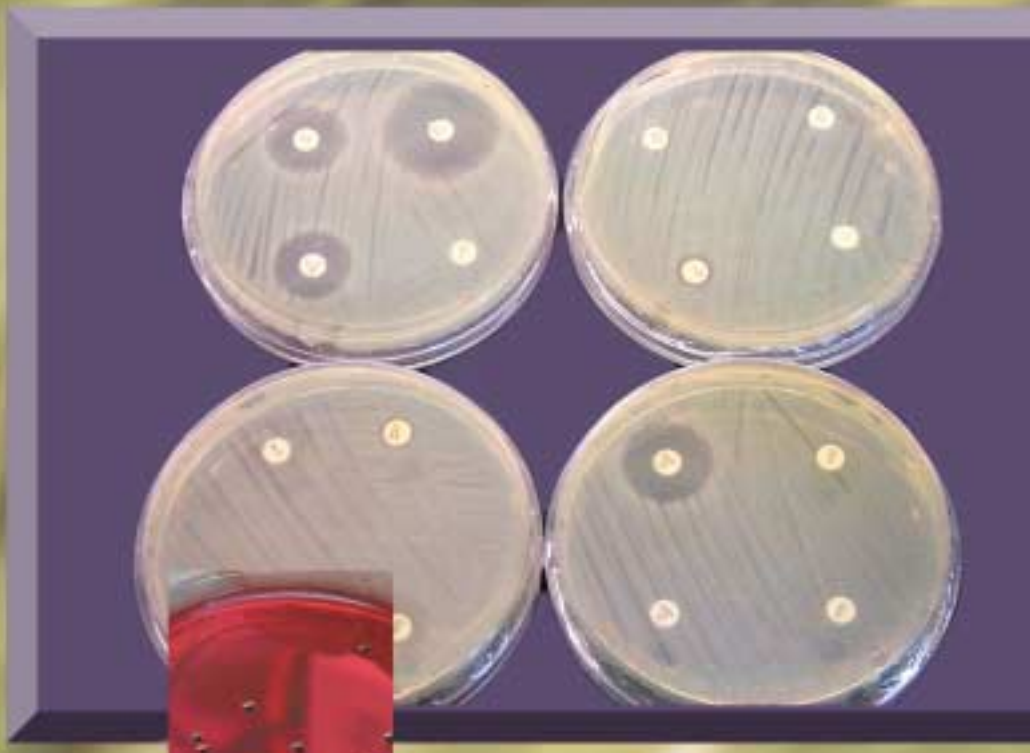
Antibiotic Resistance in Foodborne Pathogens



The National Food Centre

RESEARCH & TRAINING FOR THE FOOD INDUSTRY

RESEARCH REPORT NO 76

ANTIBIOTIC RESISTANCE IN FOODBORNE PATHOGENS

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SUMMARY

Wide-spread antibiotic resistance among bacterial pathogens is now a serious public health issue and multi-antibiotic resistance has been reported in many foodborne pathogens including *Salmonella* and *E. coli*.

A study to determine antibiotic resistance profiles of a range of *Salmonella* and Verocytotoxigenic *E.coli* (VTEC) isolated from Irish foods revealed significant levels of antibiotic resistance in the strains. *S.* typhimurium DT104 were multi-antibiotic resistant with 97% resistant to 7 antibiotics. *S.* Dublin and *S.* Agona showed lower levels of antibiotic resistance. Antibiotic resistance among VTEC isolates was generally low but two isolates of *E. coli* O157:H7 from minced beef were shown to be multi-antibiotic resistant (8 to 10 antibiotics).

Studies to determine the relationship between antibiotic resistance and tolerance to heat were conducted. The survival of *Salmonella* spp. with different antibiotic profiles (antibiotic sensitive, laboratory-developed antibiotic resistant mutants or multi-antibiotic resistant) on chicken heated to 55°C with or without a prior heat shock at 48°C was established. The D value (time in minutes to achieve a 90% or 1 log reduction in the *Salmonella* population) recorded for a multi-antibiotic resistant *S.* Typhimurium DT104 was significantly higher ($P<0.05$) than for all other strains tested (antibiotic sensitive or with laboratory-acquired antibiotic resistance). These results suggest that the presence of multi-antibiotic resistance genes increased the heat tolerance of *Salmonella*.

The survival of VTEC, *E. coli* O157:H7 and *E. coli* O26 (antibiotic sensitive, laboratory-developed antibiotic resistant mutants or multi-antibiotic resistant) in minced beef heated to 55°C with or without a prior heat shock at 48°C was established. The D value recorded for multi-antibiotic resistant *E. coli* O157:H7 was significantly lower than for the other strains tested. These results indicate that the presence of multi-antibiotic resistant genes rendered the pathogens more sensitive to heat.

Studies to determine whether antibiotic resistance could be transferred within species (*Salmonella* Typhimurium to *Salmonella* Agona) and between species

(*Salmonella* to *E. coli*) when co-inoculated into laboratory media, milk or minced beef at different storage temperatures (37, 25, 15 and 4°C) showed that horizontal antibiotic resistance gene transfer occurred in all substrates at temperatures of 37 and 25°C. At 15°C, transfer of resistance occurred only in minced meat. Similar results were reported for transfer of antibiotic resistance both within the *Salmonella* spp. and between different species (*Salmonella* to *E. coli*).

INTRODUCTION

In recent years, public health concerns regarding the occurrence of antibiotic resistance in pathogenic bacteria has increased. While antibiotics have proven to be an effective means for the prevention and control of bacterial infection, their widespread use in food animals can contribute to the selection of antibiotic resistant microbial populations in the food chain. The occurrence of antibiotic resistant strains and in particular multi–antibiotic resistant strains of foodborne pathogenic bacteria, such as *Salmonella* spp., may cause difficulties in treating illness related to these strains. A further public health concern is that the genes encoding for antibiotic resistance can potentially be transferred between bacteria, leading to the wider dissemination of antibiotic resistant genes and bacteria in the environment and in the food chain.

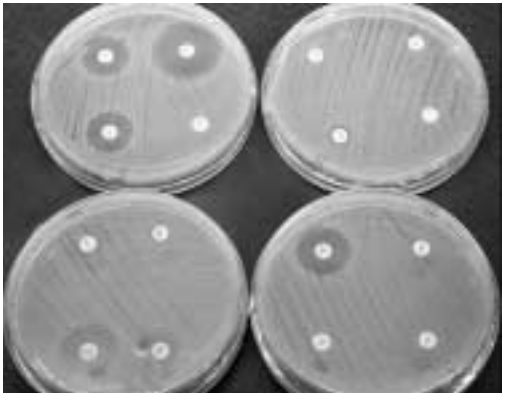
Recent research also indicates that the possession of antibiotic resistant genes, in particular multi-antibiotic resistance, may affect the survival of organisms when subjected to stresses such as heat or low pH. This may impact on how these pathogens survive under typical control measures employed in food processing and could potentially have an impact on process safety margins.

The aim of this project was to conduct an in-depth study on antibiotic resistance in two selected foodborne pathogens *Salmonella* and Verocytoxigenic *E. coli* (VTEC). Initial studies were undertaken to assess the level of antibiotic resistance in strains of these pathogens isolated from Irish foods. Selected strains of *Salmonella* and VTEC with different antibiotic resistance profiles were then inoculated into either chicken or beef, subjected to heat treatments and their survival compared. Finally studies were

conducted to establish if antibiotic resistance could be transferred within species (*Salmonella* Typhimurium to *Salmonella* Agona) and between species (*Salmonella* to *E. coli*) when present simultaneously in foods (milk, beef) stored at different temperatures.

ANTIBIOTIC RESISTANCE PROFILES

Antibiotic resistance profiles for strains of *Salmonella* spp. and VTEC isolated from animal faeces, food and clinical specimens in Ireland were determined using an agar disc diffusion technique. This involved inoculating a single colony of the selected organism from a Tryptone Soya Agar plate (TSA) into 10 ml of Mueller Hinton broth, incubating the culture overnight at 37°C and preparing a 1:10 dilution of the overnight culture in 0.1 % peptone water (Oxoid). A sterile swab was immersed in the diluted suspension and swabbed over the entire surface of a Mueller Hinton Agar plate. The plates were held at room temperature for 10 minutes and disks (MAST Diagnostics, UK) impregnated with different antibiotics were dispensed on the surface of the agar using an automatic disc dispenser (MAST Diagnostics, UK). The Mueller Hinton plates were then incubated at 37°C for 18-24 h and examined for zones of inhibition in growth of the organism around each antibiotic (Photo 1). The size of the zone of inhibition was recorded to the nearest millimetre. Resistance among both VTEC and *Salmonella* isolates to a range of 16 different antibiotics was tested.



A zone of inhibition around the disc indicates the organism is sensitive to the antibiotic; no zone indicates antibiotic resistance

Salmonella spp

The results showed a difference in antibiotic resistance profiles between the different strains of *Salmonella* (Table 1). Among *S.* typhimurium DT104 (n=78), 97% were resistant to 7 antibiotics. Among *S.* Dublin isolates (n=9), 11% were resistant to 4 antibiotics and 56% to 3 antibiotics while among *S.* Agona (n=6), 100% of strains were resistant to 2 antibiotics. Among *S.* Typhimurium DT104 isolates, resistance to ampicillin and chloramphenicol was 100%. This combination of resistance in *Salmonella* is particularly undesirable in terms of human illness as these two antibiotics are the drugs of choice in the clinical treatment of salmonellosis and a wide range of other bacterial infections.

VTEC

All *E.coli* O157:H7 strains tested were resistant to rifampin and three of the strains (all beef isolates) were resistant to ≥ 4 antibiotics, including 2 from minced beef which were multi-resistant (8-10 antibiotics) (Table 2). Antibiotic resistance profiles for isolates of *E. coli* O111 and *E. coli* O26 showed rifampin resistance in all strains. One strain of *E. coli* O111 was resistant to 4 antibiotics. Antibiotic resistant VTEC are of limited clinical significance; the use of antibiotics in the treatment of infection is controversial since, depending on the stage of infection, they may increase the level of free verotoxin *in vivo*, thus facilitating disease progression. However, knowledge of antibiotic resistance patterns in VTEC is important to enable an overview of the contribution that they make to the antibiotic resistance problem among foodborne bacteria.

Table 1. Antibiotic resistance (%) among *Salmonella* isolates

Antibiotics	*S.* Typhimurium DT104 (n=78)	*S.* Dublin (n=9)	*S.* Agona (n=6)
Ampicillin	100	-	-
Kanamycin	-	-	-
Cefachlor	-	11	-
Cefixime	-	-	-
Streptomycin	100	-	-
Trimethoprim	-	-	-
Nalidixic Acid	-	-	-
Rifampin	100	100	100
Sulfonamides	100	56	-
Chloramphenicol	100	-	-
Tetracycline	100	-	-
Monocycline	-	-	-
Ciprofloxacin	-	-	-
Doxycycline	-	-	-
Amikacin	1	-	-
Erythromycin	97	100	100

Table 2. Antibiotic resistance (%) among veroctyotoxigenic *E.coli* isolates

Antibiotics	*E. coli* 157:H7 Beef abattoir isolates (n=204)	*E. coli* O157:H7 Retail Meat Isolates (n=45)	*E. coli* O157:H7 Clinical Isolates (n=8)	*E. coli* O111 Various* (n=16)	*E. coli* O26 Various* (n=15)
Ampicillin	2	4	-	13	-
Kanamycin	1	2	-	13	-
Cefachlor	2	2	-	-	-
Cefixime	4	2	-	-	-
Streptomycin	9	36	-	25	-
Trimethoprim	0.5	2	-	6	-
Nalidixic Acid	3	27	-	-	-
Rifampin	100	100	100	100	100
Sulfonamides	13	7	-	19	-
Chloramphenicol	0.5	2	-	-	-
Tetracycline	2	9	-	6	13
Minocycline	2	2	-	13	-
Ciprofloxacin	-	-	-	-	-
Doxycycline	-	9	-	6	-
Norfloxacin	0.5	-	-	-	-
Moxalactam	0.5	-	-	-	-

* Isolates from various clinical and veterinary sources.

TOLERANCE

Salmonella

Research was carried out to elucidate the relationship, if any, between the presence of antibiotic resistance genes in strains of *Salmonella* (isolated from Irish foods) and their tolerance to heat.

Chicken pieces were inoculated with strains of *S.* Enteritidis or *S.* Typhimurium which were antibiotic sensitive (AS); counterparts of these sensitive strains with laboratory-developed antibiotic resistance (AR) or multi-antibiotic resistant *S.* Typhimurium DT1O4 (MR). Half of the chicken samples were heat shocked (48°C for 30 min) and all were then heat challenged at 55°C for up to 30 min. Samples were removed from the heat at various time intervals during the 30 minute period and plated onto Tryptone Soya Agar (TSA), overpoured with XLD and the colonies were counted. The number of survivors was plotted against heating time and the D value calculated (time in minutes to achieve a 90% or 1 log reduction in the *Salmonella* population) (results shown in Table 3). Heat-shocked strains of *S.* Typhimurium DT104 had significantly higher D values than their non-heat-shocked counterparts. No significant differences in D values were observed between antibiotic sensitive strains of *S.* Enteritidis and *S.* Typhimurium and their antibiotic resistant counterparts. However, the D value at 55°C (D_{55}) for multi-antibiotic resistant *S.* Typhimurium DT104 (6.33 min) was significantly higher ($P<0.05$) than for all other strains. These results indicate that multi-antibiotic resistance influenced the heat tolerance of *Salmonella*. These results are significant when considering thermal treatment for foods and safety margins should take account of possible increased heat tolerance among strains of *Salmonella* which may be multi-antibiotic resistant.

Table 3. D value at 55°C (min) for heat shocked (48°C/ 30 min) and non-heat shocked *Salmonella* strains with different antibiotic profiles on chicken meat.

Salmonella Serovar	Heat treatment	D value at 55°C (min)
S. Enteriditis AS	No Heat Shock	3.58
S. Enteriditis AS	Heat Shock	4.50
S. Enteriditis AR	No Heat Shock	3.64
S. Enteriditis AR	Heat Shock	5.00
S. Typhimurium AS	No Heat Shock	2.52
S. Typhimurium AS	Heat Shock	3.89
S. Typhimurium AR	No Heat Shock	2.74
S. Typhimurium AR	Heat Shock	4.07
S. Typhimurium DT104 MR	No Heat Shock	3.80
S. Typhimurium DT104 MR	Heat Shock	6.33

AS = antibiotic sensitive; AR = antibiotic resistant; MR = Multi-antibiotic resistant

Verocytotoxigenic E.coli

This study aimed to elucidate the relationship between the presence of antibiotic resistance in strains of VTEC and their tolerance to heat.

E.coli O157:H7 and *E. coli* O26 strains which were antibiotic sensitive (AS), counterparts of these strains with laboratory-developed antibiotic resistance (AR) or multi-antibiotic resistant (MR) (resistant to10 antibiotics) were each inoculated into minced beef samples. Half of the meat samples were heat shocked (48°C for 30 min) and all were heat challenged at 55°C for up to 1 h. Samples were removed from the heat treatment at various time intervals during the 30 minute period and plated onto Tryptone Soya Agar (TSA),

overpoured with CT-SMAC / CT-RMAC and the colonies were counted. The number of survivors was plotted against heating time and the D value calculated (results shown Table 4). All heat-shocked strains had higher D-values then their counterpart non-heat-shocked strains.

No significant differences in D values were observed between antibiotic sensitive strains of *E. coli* O157:H7 and *E.coli* O26 and the antibiotic resistant (AR) counterparts of these strains. However, the D_{55} value for multi-antibiotic resistant *E. coli* O157:H7 (MR) (1.51 –1.71 min) was significantly lower than for any of the other strains examined ($P < 0.05$) (D value 9.73 - 13.15 min). These results indicate that the presence of multi-antibiotic resistant genes significantly weakened the heat tolerance of VTEC, which is the converse of that observed in *Salmonella*.

Table 4. D values (min) for *E.coli* O157:H7 and *E.coli* O26 (with varying antibiotic resistance profiles) in minced meat heated at 55°C with or without a prior heat shock at 48°C for 30 min

E. coli Serovar	Heat treatment	D value at 55°C (min)
E. coli O157 AS	No Heat Shock	11.70
E. coli O157 AS	Heat Shock	13.15
E. coli O157 AR	No Heat Shock	11.36
E. coli O157 AR	Heat Shock	11.90
E. coli O157 MR	No Heat Shock	1.71
E. coli O157 MR	Heat Shock	1.51
E. coli O26 AS	No Heat Shock	9.73
E. coli O26 AS	Heat Shock	12.19
E. coli O26 AR	No Heat Shock	8.64
E. coli O26 AR	Heat Shock	11.14

AS = Antibiotic sensitive; AR = Antibiotic resistant, MR = multi-antibiotic resistant

Overall, the results show a link between antibiotic resistance and heat tolerance in *Salmonella* and VTEC though converse results were observed in each species with heat tolerance and sensitivity conferred respectively to these pathogens. The reason for this phenomenon is unclear and further study is needed with more multi-antibiotic resistant strains to see if this phenomenon is widespread among these phenotypes. Research is also needed to understand at a genetic/ molecular level the cross reaction between antibiotic resistance and resistance/tolerance to other stresses which is occurring in these pathogens.

TRANSFER OF ANTIBIOTIC RESISTANCE BETWEEN BACTERIA

It is well known that many foodborne bacteria carry genes that confer antibiotic resistance on mobile genetic elements which have the ability to leave and enter bacterial cells. However little research has been conducted on the horizontal transfer of genes that encode for antibiotic resistance between pathogens in foods under typical storage conditions. This study was carried out to investigate if antibiotic resistance could be transferred between related strains of *Salmonella* (S. Typhimurium to *S.* Agona) and between different bacterial species (*Salmonella* to *E. coli*) under laboratory simulated occasions (broth) in milk and in minced beef at different storage temperatures.

Transfer of resistance from *S.*Typhimurium to *S.* Agona or *E.coli* K12

S. Typhimurium which was ampicillin resistant but sensitivite to nalidixic acid was selected as a donor strain. A recipient *S.* Agona strain and an *E.coli* K12 were rendered chromosomally-resistant to nalidixic acid (NA). Chromosomal borne antibiotic resistance is not transferable.

The donor (*S.* Typhimurium) and recipient strains (*S.* Agona or *E.coli* K12) were grown up separately in 9 ml Millers Luria Bertani broth (LB) for 18h at 37°C. An inoculum containing ~ 10^7 ($\log_{10}7.0$) cfu ml^{-1} of each strain (1:1 ratio) was then added to Millers LB broth (100ml), milk (100ml) or minced

beef (100g) and incubated for 18 h at 4, 15, 25 or 37°C. Following incubation, 100 µl was plated in duplicate onto Tryptone Soya Agar (TSA) (Oxoid) with the appropriate combination of antibiotics to identify the donor, recipient and transconjugants (an *S. agona* or *E. coli* K12 which has acquired resistance to ampicillin from *S.* Typhimurium). The plates were then overpoured with Xylose Lysine Decarboxylase (XLD) to select for *Salmonella* isolates only or with McConkey–3 (Oxoid) to select for *E.coli* isolates only and then incubated for 24h at 37°C. The results are presented in Tables 5 and 6. Antibiotic resistance transfer occurred in all matrices investigated (LB broth, pasteurised milk and minced beef) at 37 and 25°C. But at 15°C it occurred only in minced beef while at 4°C no transfer was observed. Similar results were observed regardless of whether the recipient cell was of the same or different species (*E.coli* K12).

Overall the results of this study demonstrate that within a food environment, bacteria can potentially exchange genetic material conferring antibiotic resistance. This occurs not only between bacteria of the same species (*Salmonella* to *Salmonella*) but also between species (*Salmonella* to *E. coli*) indicating that the genes conferring resistance are very mobile and demonstrates clearly how readily antibiotic resistance can be spread throughout the food environment. The reduced occurrence of antibiotic resistance transfer at lower temperatures indicates that, in foods stored under chill conditions, antibiotic resistance transfer will be minimised.

Table 5: Number of *Salmonella* Typhimurium (donor cells), *S.* Agona (recipient cells) and *S.* Agona transconjugants (*i.e.* with acquired antibiotic resistance) following incubation in LB broth, milk or minced beef for 24 or 48 h.

Length of time in medium and storage temperature	Donor (log_{10}cfu ml^{-1})	Recipient (log_{10}cfu ml^{-1})	Transconjugant (log_{10}cfu ml^{-1})	Ratio of transconjugant to recipient (log_{10}cfu ml^{-1})
L.B Broth after 24 h				
25°C	9.50	8.91	3.59	1.20 x 10-6
37°C	9.79	8.98	4.91	1.31 x 10-5
Milk after 24 h				
25°C	8.42	8.69	2.46	1.0 x 10-6
37°C	9.53	9.44	3.72	1.5 x 10-6
Minced beef after 24 h				
15°C	7.96	7.59	2.64	4.7 x 10-6
25°C	8.33	8.16	3.74	2.57 x 10-5
37°C	8.52	8.45	3.91	2.45 x 10-5
Minced beef after 48 h				
15°C	8.14	7.79	2.45	2.0 x 10-6
25°C	8.42	8.26	4.11	4.9 x 10-5
37°C	8.47	8.13	4.04	3.71 x 10-5

Note: At 4°C there was no transfer of antibiotic resistance in any substrate and at 15°C there was no transfer of resistance in either broth or milk

Table 6 Number of *Salmonella* Typhimurium (donor cells), *E. coli* (recipient cells) and *E.coli* transconjugants (i.e. with acquired antibiotic resistance) following incubation in LB broth, milk or minced beef for 24 or 48 h.

Length of time in medium and storage temperature	Donor (log_{10}cfu ml^{-1})	Recipient (log_{10}cfu ml^{-1})	Transconjugant (log_{10}cfu ml^{-1})	Ratio of transconjugant to recipient (log_{10}cfu/ml^{-1})
L.B Broth after 24 h				
25ºC	7.69	7.67	4.13	2.75 x 10-4
37ºC	7.56	7.69	4.45	7.8 x 10-4
Milk after 24 h				
25ºC	8.34	6.75	3.17	6.7 x 10-6
37ºC	8.75	7.61	4.07	2.1 x 10-5
Minced beef after 24 h				
15ºC	7.37	5.92	0.22	7.08 x 10-8
25ºC	7.82	6.26	1.93	1.20 x 10-6
37ºC	7.77	6.58	3.49	5.24 x 10-5
Minced beef after 48 h				
15ºC	7.70	5.82	0.52	6.61 x 10-8
25ºC	8.10	6.32	3.08	9.50 x 10-6
37ºC	8.11	5.71	4.23	1.31 x 10-4

Note: At 4ºC there was no transfer of antibiotic resistance in any substrate and at 15ºC there was no transfer of resistance in either broth or milk

CONCLUSIONS

The study indicated that there were high levels of antibiotic resistance and multi-antibiotic resistance among foodborne *Salmonella* isolates. In VTEC the level of antibiotic resistance was generally low, although some multi-antibiotic resistant strains of *E. coli* O157:H7 from minced beef were detected.

The studies showed a link between the presence of multi-antibiotic resistance (MR) genes in an organism and its tolerance to heat. In *Salmonella*, MR made the organism more heat resistant while in the case of *E. coli* O157 the cell became less heat resistant. The reason for this requires further examination at a molecular level.

Studies on the transfer of antibiotic resistance between bacteria co-inoculated in to the same food samples indicated that when conditions were favourable, *i.e.* at higher temperatures (37 and 25°C), genes conferring antibiotic resistance could be transferred between organisms. In meat this also occurred at 15°C.

Overall the results of this study established that antibiotic resistance, in particular multi-antibiotic resistance, is a significant issue in foodborne bacteria, including *Salmonella* and *E. coli*. Not only is there a considerable prevalence of antibiotic resistance, in particular multi-antibiotic resistant organisms, in the food environment but the acquisition of these genes alters the reaction of the cell to controls which are commonly employed in the food industry such as heat. Genes conferring resistance can be transferred between cells at ambient temperatures (25°C) and at inadequate refrigeration temperatures (15°C). This indicates that the problem of antibiotic resistance can be transferred throughout the micro-flora in the food chain and that the problem will continue to grow. Further research on multi-antibiotic resistance and its effect on the cell at a molecular level is required.

RECOMMENDATIONS

- This study demonstrated a potential link between antibiotic resistance and tolerance to heat among foodborne pathogenic bacteria. When designing and validating the safety of a particular product, trials should be conducted using micro-organisms with a proven high level of resistance to foodborne stress to ensure that adequate safety margins are incorporated into the process. Among *Salmonella spp.*, serovar DT104 (multi-antibiotic resistant) appears to be more tolerant to food processing stresses than other serovars and so would be a good choice for use in safety validation trials. Among *E. coli* O157:H7, strains which are multi-antibiotic resistant appear to be highly sensitive to foodborne stress and therefore would be unsuitable for use in food safety validation trials.

- Storage of foods at a chill temperature limits the potential for spread of antibiotic resistance among the microbial flora in the food.

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