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Survival of *Escherichia coli* O157:H7 in bottled natural mineral water

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M. KERR, M. FITZGERALD, J.J. SHERIDAN, D.A. MC DOWELL AND I.S. BLAIR. 1999. A non-verotoxin-producing isolate of *Escherichia coli* O157:H7 was inoculated at final concentrations of 10^3 or 10^6 ml^{-1} into natural non-carbonated mineral water (MW), sterile natural mineral water (SMW) and sterile distilled deionized water (SDDW) and stored at 15 °C for 10 weeks. Samples were examined every 7 d for the presence of *E. coli* O157:H7 using a resuscitative/selective agar procedure. The MW samples were also plated onto a non-selective agar, R2A, to enumerate *E. coli* O157:H7 and the autochthonous flora. There was a significant difference in the survival of *E. coli* O157:H7 (10^3 ml^{-1} inoculum) between the MW and the SDDW at time periods 0, 7, 14 ($P < 0{\cdot}005$) 21, 28, 35 ($P < 0{\cdot}001$) and 42 d ($P < 0{\cdot}05$) and between the MW and the SMW at time periods 7, ($P < 0{\cdot}05$) 14, 21 ($P < 0{\cdot}005$) 28 ($P < 0{\cdot}01$) and 35 d ($P < 0{\cdot}05$), with the pathogen surviving longest in the MW samples. In contrast, at 10^6 ml^{-1}, no significant differences in the survival of *E. coli* O157:H7 were observed between the water types. The presence of *E. coli* O157:H7 (10^3 ml^{-1}) in the MW samples did not have an antagonistic effect on the recovery of the autochthonous flora. Transmission electron microscopy analysis demonstrated that the *E. coli* O157:H7 cells lyse during storage, releasing their contents into the surrounding environment. These substances may have been utilized by the autochthonous flora and thereby explain why the numbers of flora recovered from the inoculated MW samples were higher than those recovered from the uninoculated samples.

INTRODUCTION

Escherichia coli O157:H7, first identified as a human pathogen in 1982 by its association with two outbreaks of haemorrhagic colitis (Riley *et al.* 1983), is now considered a serious threat to public health, with some forms of infection having very severe clinical implications (Padhye and Doyle 1992). *Escherichia coli* O157:H7 has been implicated in outbreaks in a wide range of foodstuffs, such as apple cider (Besser *et al.* 1993), yoghurt (Morgan *et al.* 1993), untreated milk (Chapman *et al.* 1993), beef-related products (Bell *et al.* 1994) and cooked roast pork (Ahmed 1997). Person to person spread can occur, suggesting that the infective dose is low (Willshaw *et al.* 1994; Tilden *et al.* 1996; Easton 1997). Outbreaks due to the consumption of water containing *E. coli* O157:H7 have occurred in the USA (Swerdlow *et al.* 1992; Keene *et al.* 1994), South Africa and Swaziland (Isaacson *et al.* 1993) and Scotland (Dev *et al.* 1991). While many of these cases are related to the consumption of contaminated surface waters, there is now increasing concern that the entry of this organism into groundwater supplies may pose risks in relation to the consumption of bottled waters.

The past decade has seen a dramatic increase in the consumption of bottled natural mineral waters in the European Community, to a point where consumption exceeds that of carbonated soft drinks (Green and Green 1994). This increase in demand may principally be driven by changes in fashion towards the conspicuous consumption of 'designer water', but it may also reflect increased consumer concerns in relation to perceptions about the safety of mains supply water (Hunter 1994). Whatever the reasons for the growth in the con-

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sumption of bottled mineral waters, adequate knowledge about the fate of pathogens that may gain access to mineral water sources is essential to enable suppliers to assure public health surveillance agencies and consumers of the quality and safety of the product.

The purpose of the present study was to evaluate the survival of *E. coli* O157:H7 in bottled natural mineral water. In this investigation the survival of *E. coli* O157:H7 was compared in natural non-carbonated mineral water (MW), in the same water after filtration which removed the autochthonous flora (SMW) and in sterile distilled deionized water (SDDW).

MATERIALS AND METHODS

Bacterial strain

A non-verotoxin-producing strain of *E. coli* O157:H7 (NCTC 12900) was obtained from the National Collection of Type Cultures (Central Public Health Laboratory, Colindale, London, UK) and maintained on Protect beads (Technical Consultant Services, Heywood, Lancaster, UK) at −20 °C.

Water samples

Three types of water were used.

1 A European brand of bottled non-carbonated natural MW which had been bottled 9–12 months previously in 1·5-l polyethylene terephthalate bottles was used in this experiment. The bottled MW had been stored at between 10 and 15 °C in an Irish warehouse which distributes the MW to retail outlets. Two to four weeks prior to commencement of the study, three different batches of MW were obtained from the warehouse and stored at 2 °C in the laboratory. On the day of inoculation the MW was brought to room temperature (15–20 °C).
2 Sterile non-carbonated natural MW was prepared by filter-sterilizing an aliquot of the above MW through a 0·2-μm filter (Sartorius, Goettingen, Germany). This process was carried out the day before use to remove the autochthonous flora present. The SMW was stored at room temperature (15–20 °C) prior to inoculation.
3 Sterile distilled deionized water; mains supply water was distilled using a Fisons distillation unit and deionized using an ElgastatB114 unit (Fison, Wycombe, UK). The water was autoclaved at 121 °C for 15 min and filter-sterilized through a 0·2-μm filter (Sartorius) to remove particulate matter. This process was carried out the day before use and the SDDW was stored at room temperature (15–20 °C) prior to inoculation.

Media

The media used in this study were obtained from Oxoid (Basingstoke, UK) and prepared in accordance with the manufacturer's instructions. The prepared media were stored at 4 °C and brought to room temperature prior to use. Media used included Brain Heart Infusion (BHI) Broth (CM 225), Tryptone Soya Agar (TSA; CM 131), Sorbitol MacConkey Agar (SMAC; CM 813)) and R2A Agar (CM 906). The media used to enumerate the bacteria in the MW and SMW samples were prepared in non-carbonated MW. The media used to enumerate the *E. coli* O157:H7 in the SDDW samples were prepared in distilled deionized water. A previous study by Morais and da Costa (1990) had shown that media prepared in MW enhanced the recovery of the autochthonous flora from MW.

Inoculum preparation

A Protect bead containing non-toxin-producing *E. coli* O157:H7 cells was removed from storage at −20 °C and inoculated into 20 ml BHI broth prepared in MW and incubated at 37 °C for 24 h. An aliquot of the resultant culture (1 ml) was transferred to 150 ml fresh BHI broth and incubated at 37 °C for 19 h. Previous studies had established that such cultures of *E. coli* O157:H7 were in the late stationary phase of growth. Two separate aliquots (25 ml) of the bacterial suspension were placed into two centrifuge tubes and centrifuged at 2800 *g* for 10 min at 4 °C (centrifuge 5403; Eppendorf, Hamburg, Germany). The supernatant fluid was discarded from the cell pellets formed and one pellet was washed three times by resuspension and recentrifugation in SMW. The other pellet was washed three times by resuspension and recentrifugation in SDDW.

Standardization of *Escherichia coli* O157:H7 inocula

The numbers of *E. coli* O157:H7 cells in the washed cell suspensions were determined by preparing a 10-fold dilution series of each, in 9-ml aliquots of filter-sterilized MW or SDDW as appropriate. The membrane filtration epifluorescent technique was used for enumeration. Briefly, 1 ml of each dilution was pipetted onto, and vacuum-filtered through, a 25-mm, 0·6-μm polycarbonate membrane (Poretics Products, Osmonics, CA, USA). The membrane was flooded with 1 ml 0·025% (w/v) acridine orange solution for 30 s and excess stain removed by washing the membrane while still under vacuum with 0·1 ml industrial methylated spirits. Each membrane was air-dried and mounted in a non-fluorescent immersion oil (Nikon, Fuji, Tokyo, Japan) on a glass slide beneath a cover-slip. The stained preparations were examined under u.v. light (wavelength range 450–490 nm) using a 100× oil immersion objective on a

fluorescent microscope (Nikon) with an epifluorescent attachment and a 100 W mercury vapour lamp as a light source. Counts were determined by visual inspection and the numbers of fluorescing *E. coli* O157: H7 cells in 20 different fields of vision for each membrane. From these counts the numbers of cells ml^{-1} in the original suspensions were calculated using a working factor of 981·25 (Duffy and Sheridan 1997).

Inoculation of water samples for survival studies

Using the most appropriate dilutions, the three types of water, MW, SMW and SDDW, were inoculated with *E. coli* O157:H7 to achieve two different inoculum levels, 10^3 and 10^6 ml^{-1}. The samples were aliquoted out in 10-ml amounts into 30-ml plastic sterile universal bottles (Sterilin) and stored at 15 ± 1 °C for 70 d. This temperature was chosen because it best represented the temperature at which MW is stored in most retail outlets (10–20 °C). Uninoculated control samples were also included in the study. The experiment was performed on three separate occasions, a different batch of MW being used for each replicate.

Inoculation of water for transmission electron microscopy studies

A separate experiment was set up to assess the ultrastructural changes occurring to the *E. coli* O157:H7 cells following storage in MW at 15 °C. Mineral water (11 l) was inoculated as described above, to achieve a cell density of 10^6 *E. coli* O157:H7 cells ml^{-1}, and stored at 15 °C.

Enumeration of *Escherichia coli* O157:H7 in water samples

A separate set of samples was removed from storage every week for 10 weeks. A 10-fold dilution series in SMW was prepared from inoculated MW and SMW samples and their respective uninoculated controls. A 10-fold dilution series in SDDW was prepared from inoculated SDDW samples and the uninoculated SDDW controls. The numbers of surviving *E. coli* O157:H7 cells in the samples and in members of each dilution series were determined in duplicate, using the spread plate technique in which two 0·1-ml volumes of sample/dilution were spread over the surface of TSA plates using a sterile glass rod. After spreading, the plates were incubated at 25 °C for 6 h to allow recovery of stressed cells and then overlaid with 10–15 ml molten SMAC agar cooled to 50 °C and incubated at 37 °C for 18 h.

Enumeration of *Escherichia coli* O157:H7 and the autochthonous flora in mineral water

Both *E. coli* O157:H7 and the autochthonous flora present in the MW samples were enumerated on R2A agar. Since the colonial morphologies of *E. coli* O157:H7 and the autochthonous flora were distinct it was possible to distinguish between the pathogen and the flora and, therefore, to enumerate the autochthonous flora. On the R2A agar *E. coli* O157:H7 appeared as spherical pale cream colonies 2–4 mm in diameter, slightly convex and smooth with entire edges, while the autochthonous flora formed colonies which were smaller (0·5–2·0 mm) and paler; many were pin-point colonies. Furthermore, a random selection of typical *E. coli* O157:H7 colonies was confirmed using the Wellcolex *E. coli* O157:H7 kit (Murex Diagnostics, Chatillon, France).

Using the above MW samples and dilutions, two sets of plates were plated in duplicate by pipetting two 0·1-ml volumes on plates of the non-selective agar R2A and spreading on the surface using a sterile glass rod. One set of plates was incubated at 37 °C for 2 d and the other at 22 °C for 3 d. The SMW and SDDW uninoculated samples were also plated on R2A agar using the procedure outlined above to ensure sample sterility.

Recovery of bacteria from stored water samples for transmission electron microscopy studies

Samples for transmission electron microscopy (TEM) were withdrawn from the inoculated bulk volumes as follows: d 0 (1 l), d 13 (3 l), d 33 (3 l) and d 58 (4 l) and filtered through a 47-mm diameter, 0·2-μm pore size cellulose nitrate filter (Sartorius). The filter was vortex-mixed for 30 s with 20 ml SMW to resuspend the bacteria from the filter. The filter was aseptically removed and the bacteria harvested by centrifuging at 2800 *g* for 15 min (centrifuge 5403; Eppendorf). The supernatant fluid was discarded and the pelleted cells resuspended and fixed for 1·5 h in 3% (w/v) gluteraldehyde prepared in 0·5 mol l^{-1} phosphate buffer. The suspension was transferred into a sterile Eppendorf tube and the cells recovered from the fixing solution by centrifugation at 10 300 *g* (centrifuge S417-R; Eppendorf) for 15 min. The resultant pellet was washed six times by centrifugation to remove unreacted gluteraldehyde and resuspended in 0·5 mol l^{-1} phosphate buffer. The washed pellet was post-fixed in 2% (w/v) osmium tetroxide prepared in 0·5 mol l^{-1} phosphate buffer for 1 h. The fixed pellet of cells was dehydrated in a graded series of ethanol/water mixtures, ranging from 10 to 100% ethanol. The dehydrated pellet of cells was transferred through two changes of propylene oxide for 15 and 30 min, respectively. The pellet was then placed in a mixture of 50% propylene oxide and 50% epoxy resin (agar 100 resin; Agar Scientific, Cambridge, Stanstead, UK) for 2 h, before being immersed in 100% epoxy resin for a further 2 h. The pellet of cells was placed in an embedding mould containing fresh 100% epoxy resin and transferred to a vacuum embedding oven, evacuated to 500 mmHg and left for 20 min to remove air bubbles from the epoxy resin. After vacuum

release the embedding oven was maintained at 60 °C and atmospheric pressure for 24 h to polymerize the epoxy resin. Thin sections (700 nm) were cut from the resin block using an OMU3 Microtome (Reichert), mounted on 300 mesh copper grids and examined in an H-7000 (Hitachi, Wokingham, UK) transmission electron microscope at 100 kV accelerating voltage (Electron Microscopy Unit, Trinity College Dublin, Ireland).

Statistical analysis

In order to determine the effect of water type on the survival of *E. coli* O157:H7, analysis of variance (ANOVA) was used to compare the numbers of *E. coli* O157:H7 cells recovered on TSA/SMAC plates derived from the three water types after each sampling occasion. In addition, ANOVA was used to compare the numbers of autochthonous flora being recovered on R2A agar from the uninoculated MW samples compared with those recovered from the inoculated MW preparations both at 37 and 22 °C after each sampling occasion. The statistical package used was Genstat 5 (Rothamsted Experimental Station, Harpenden, Herts, UK).

RESULTS

Effect of water type on *Escherichia coli* O157:H7 survival

The numbers of *E. coli* O157:H7 surviving in each of the three water types during storage at 15 °C for up to 70 d are presented in Fig. 1. This shows that, at the lower inoculum level of 10^3 ml^{-1}, 0·56 $\log_{10}$ *E. coli* O157:H7 survived in MW at 63 d, but the pathogen was no longer detectable on day 70. In the SMW, 0·64 $\log_{10}$ *E. coli* O157:H7 were still detected on day 42, but no organisms were detected on day 49. In the SDDW, 0·64 $\log_{10}$ *E. coli* O157:H7 were detected on day 14, but none were detected on day 21. The differences in the survival of *E. coli* O157:H7 between the MW and SDDW were significant on days 0, 7 and 14 ($P < 0·01$), on days 21, 28 and 35 ($P < 0·001$) and on day 42 ($P < 0·05$). Comparing the survival of the pathogen between the MW and SMW significant differences were detected on days 7 ($P < 0·05$), 14, 21 ($P < 0·01$), 28 ($P < 0·01$) and 35 ($P < 0·05$). No significant differences in the survival of *E. coli* O157:H7 were observed between the SMW and the SDDW over the 70-d storage period.

At the higher inoculum level of 10^6 cells ml^{-1}, there was no significant difference in the survival of the pathogen between the three water types (Fig. 2). *Escherichia coli* O157:H7 had declined from 6·54 $\log_{10}$ to 3·00 $\log_{10}$ in MW, from 6·58 $\log_{10}$ to 3·53 $\log_{10}$ in SMW and from 6·17 $\log_{10}$ to 2·01 $\log_{10}$ in SDDW at the end of the 70-d storage period.

The effect of *Escherichia coli* O157:H7 on the autochthonous flora

Figure 3 shows the numbers of the autochthonous flora recovered over the 70-d storage period at 37 °C from the uninoculated MW and the MW inoculated with *E. coli* O157:H7 to an initial concentration of 10^3 ml^{-1}. On day 0, no colonies were enumerated on R2A agar at 37 °C from either the uninoculated or inoculated MW samples.

To determine the effect that *E. coli* O157:H7 had on the autochthonous flora in the MW samples, the numbers of autochthonous flora recovered from inoculated MW samples were compared with the numbers recovered from the uninoculated samples. Examination of samples plated on R2A agar gave higher estimates of autochthonous flora numbers in inoculated MW (10^3 ml^{-1}) samples than in the unin-

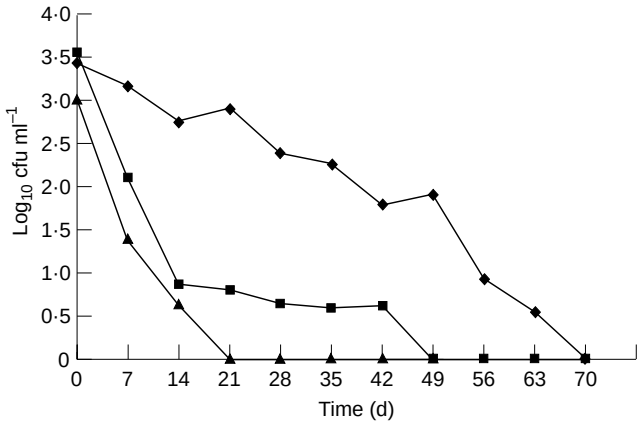

Fig. 1 Survival of *Escherichia coli* O157:H7 (10^3 ml^{-1}) in natural mineral water (◆), sterile mineral water (■) and sterile distilled deionized water (▲), when stored at 15 °C for 70 d

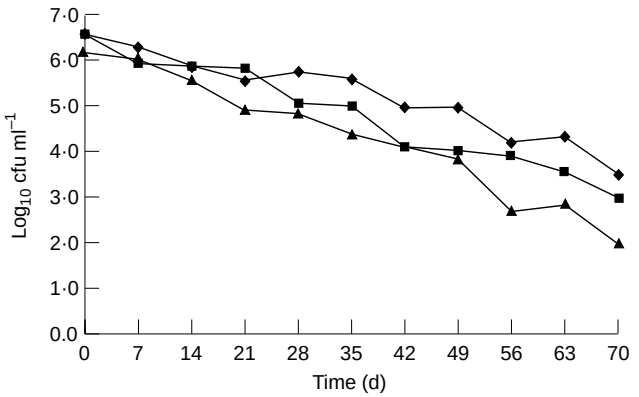

Fig. 2 Survival of *Escherichia coli* O157:H7 (10^6 ml^{-1}) in natural mineral water (◆), sterile mineral water (■) and sterile distilled deionized water (▲), when stored at 15 °C for 70 d

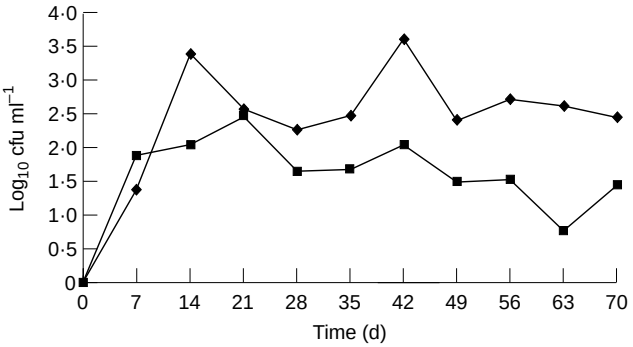

Fig. 3 Comparison of the level of autochthonous flora recovered on R2A agar at 37 °C from the natural mineral water samples (◆) inoculated with *Escherichia coli* O157:H7 (10^3 ml^{-1}) compared with uninoculated control samples (■)

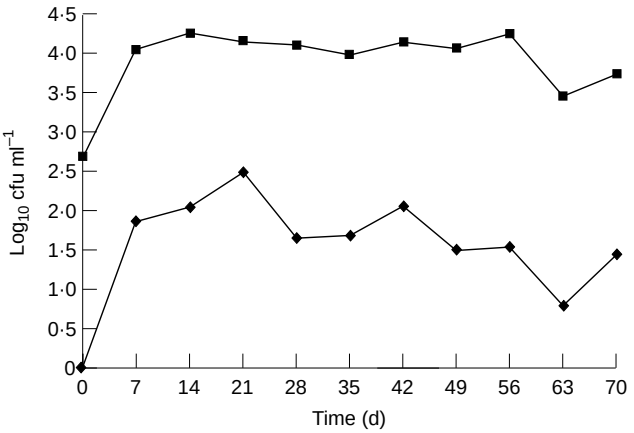

Fig. 4 The effect of incubation at 22 °C (■) and 37 °C (◆) on the recovery of the autochthonous flora on R2A agar from uninoculated natural mineral water, stored at 15 °C for 70 d

oculated MW samples. This pattern was observed on R2A agar plates incubated at both incubation temperatures, 22 and 37 °C. However, statistical analysis indicated that, in almost all cases, the apparent differences were not statistically significant.

In the case of the MW inoculated with *E. coli* O157:H7 to an initial concentration of 10^6 cells ml^{-1}, the autochthonous flora could not be accurately enumerated because the high levels of *E. coli* O157:H7 being recovered masked the lower levels of autochthonous flora present.

The effect of incubation temperature on the recovery of the autochthonous flora

Each count on the R2A agar plates prepared from MW samples (uninoculated and inoculated with *E. coli* O157:H7 to an initial concentration of 10^3 ml^{-1}) was higher at 22 °C than the related 37 °C count (Fig. 4) throughout the storage period of 70 d. There were statistically significant differences between the autochthonous flora counts at 22 and 37 °C from samples withdrawn at 0, 14, 28, 42, 56 and 63 d ($P < 0{\cdot}05$).

Visualizing ultrastructural changes to *Escherichia coli* O157:H7 cells

In order to visualize ultrastructural changes occurring in the *E. coli* O157:H7 cells following storage in MW, *E. coli* O157:H7 was inoculated into MW and examined at time intervals of 0, 13, 28 and 58 d by TEM. The cells examined on day 0 exhibited normal cell characteristics, i.e. electron-dense cytoplasm with few or no electron-transparent regions and intact cell walls and membranes (Fig. 5a). In samples withdrawn on day 13, areas of electron transparency were evident with large spaces developing between the cell wall and the cell membrane. In samples withdrawn on day 33, some *E. coli* O157:H7 cells showed the formation of protrusions in their cell walls, while others appeared to have lysed, releasing their contents into the surrounding environment (Fig. 5b). In samples withdrawn following 58 d of storage in MW, the *E. coli* O157:H7 cells were very irregular in outline with visible distortions of the cell walls and large regions of electron transparency evident in the majority of cells observed.

It should be noted that the TEM studies were performed using MW samples; therefore, apart from the inoculated *E. coli* O157:H7 cells, the autochthonous flora bacteria were also present. However, since the autochthonous flora were present at much lower levels (ranging from 0 to 3·5 $\log_{10}$ over a 58-d period in the uninoculated MW) than the *E. coli* O157:H7 cells (ranging from 6 $\log_{10}$ on day 0 to 4·2 $\log_{10}$ on day 58) and the *E. coli* O157:H7 cells tend to be larger than the autochthonous flora cells, the authors are confident that the cells depicted in the photomicrographs are *E. coli* O157:H7 cells.

DISCUSSION

This study has indicated that, at initial concentrations of 10^3 ml^{-1}, *E. coli* O157:H7 can survive for extended periods in water, surviving for up to 63 d in commercially bottled MW. At this concentration, the organism survived longer in bottled MW containing the autochthonous flora (MW, 63 d) than in MW from which the autochthonous flora had been removed (SMW, 42 d) or in SDDW (14 d). At an initial concentration of 10^6 ml^{-1}, *E. coli* O157:H7 could still be recovered from all water sample types at the end of the 70-d storage period, with 3·5 $\log_{10}$, surviving in SMW, 3·0 $\log_{10}$ in MW and 2·0 $\log_{10}$ in SDDW.

Previous studies examining the survival of *E. coli* in bottled

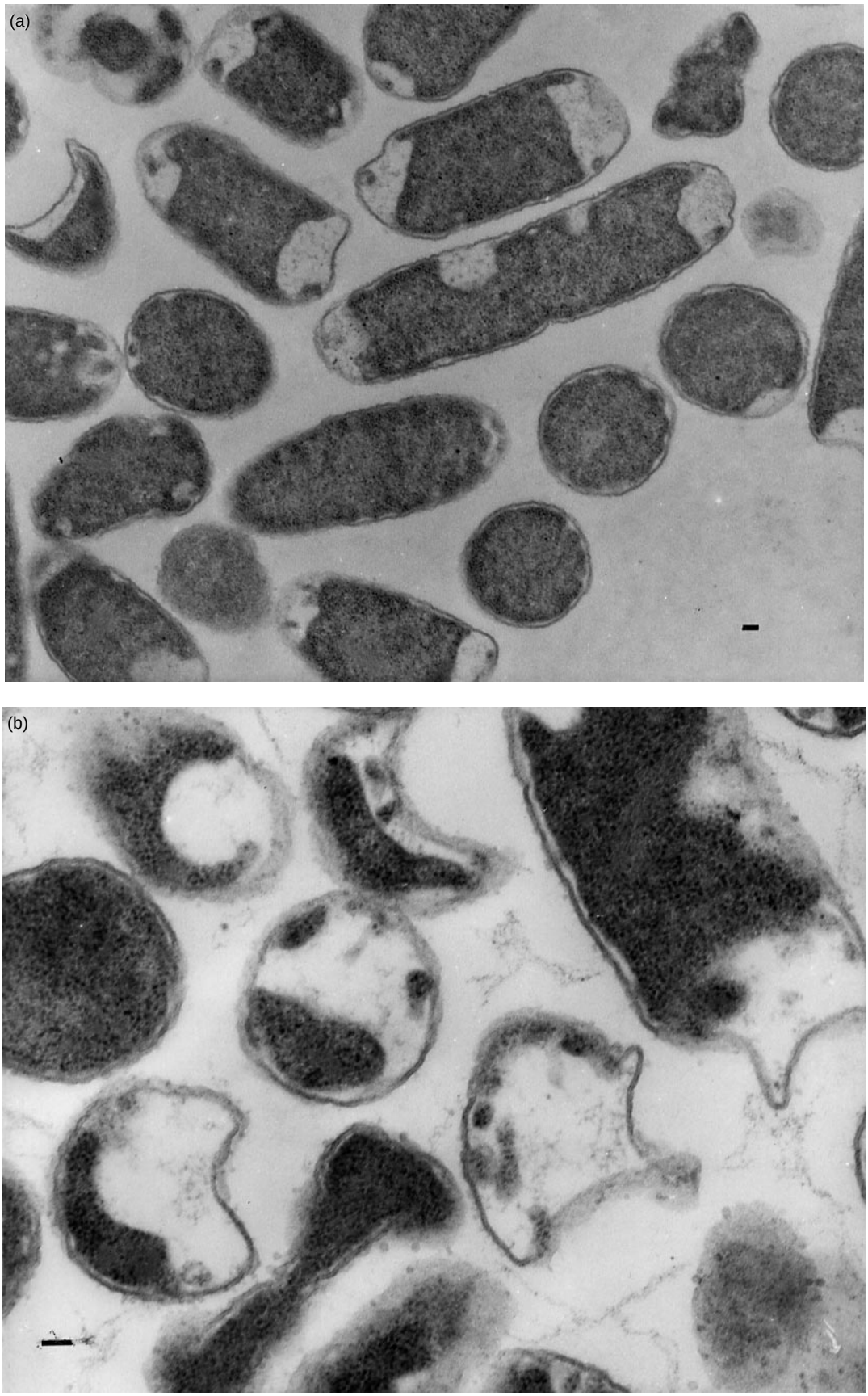

Fig. 5 Transmission electron micrographs of *Escherichia coli* O157:H7 (10^6 ml^{-1}) following inoculation into natural mineral water at 15 °C. (a) Immediately following inoculation, cells are intact and have electron-dense cytoplasm. (b) Following 33 d of storage widespread damage to the majority of the bacterial cells has occurred with large spaces beginning to appear between the cell wall and the cell membrane. Bar 100 nm

MW found that survival times for *E. coli* can vary greatly from one investigation to another. A study investigating the survival of washed *E. coli* cells in non-carbonated MW stored at 23–25 °C, using an initial inoculum of 10^4 ml^{-1}, found that the organism was not detected after 50 d (Burge and Hunter 1990). In a separate study investigating the survival of washed *E. coli* cells at inoculum levels of 10^3 and 10^7 ml^{-1} stored at 22 °C, the organism did not survive for more than 1 and 4 d, respectively (Ducluzeau *et al*. 1976). In another study, washed *E. coli* cells inoculated at a concentration of 10^6 ml^{-1} and stored at 22 °C, did not survive beyond 40 d (Lucas and Ducluzeau 1990). Comparison of these studies with the results of the present work suggests that *E. coli* O157:H7 can survive in MW longer than other strains of *E. coli.* Only one other study to date has investigated the survival of *E. coli* O157:H7 in MW (Warburton *et al.* 1998). This study differed from the present one in that, when unwashed *E. coli* O157:H7 cells were inoculated into sterile MW at 5·45 $\log_{10}$, the pathogen survived for more than 309 d. Trace amounts of nutrient from the BHI broth in which the *E. coli* O157:H7 had been grown for this study may have allowed for extended survival of *E. coli* O157:H7. The presence of even minute amounts of organic matter has been shown to extend the survival of *E. coli* in water. In a study by Ducluzeau *et al.* (1976), it was found that when 10 mg faeces was introduced into 500 ml mineral water the survival of 2 $\log_{10}$ ml^{-1} *E. coli* was extended from 1 to 50 d.

There was a significant difference in the survival of *E. coli* O157:H7 (10^3 ml^{-1}) between the MW and SDDW at different time periods (0, 7, 14, 21, 28, 35 and 42 d), indicating that the presence of the autochthonous flora in the MW significantly enhanced pathogen survival. There was also a significant difference in survival between the MW and the SMW on days 7, 14, 21, 28 and 35. The fact that, at an inoculum of 10^3 ml^{-1}, *E. coli* O157:H7 survived significantly better in MW than in either SMW or SDDW, indicates that the presence of the autochthonous flora in MW enhanced the survival of this pathogen. This is in agreement with the findings of Moreira *et al.* (1994), where it was found that *E. coli* survived better in MW than in SMW. There were no statistically significant differences between the water types at the 10^6 ml^{-1} *E. coli* O157:H7 inoculum level. The inoculum may have been so large that it overwhelmed the system and thereby masked any effects that the different water types may have had on pathogen survival.

However, other studies investigating the survival of organisms, such as *E. coli* and *Pseudomonas aeruginosa* inoculated into MW, reported that the autochthonous flora had an antagonistic effect on the survival of the inoculated organisms (Ducluzeau *et al.* 1984; Lucas and Ducluzeau 1990; Vachée *et al.* 1997). In a study by Ducluzeau *et al.* (1984) it was demonstrated that the autochthonous flora in MW had an antagonistic effect on the survival of *E. coli* due to inhibitory substances accumulating in the water from the breakdown of autochthonous flora cells. This antagonistic effect by the autochthonous flora on *E. coli* was also demonstrated in a study by Lucas and Ducluzeau (1990). In a separate study by Vachée *et al.* (1997), *E. coli*, *Staphylococcus aureus* and *Aeromonas hydrophilia* were particularly sensitive to siderophore production by isolates of MW origin. The MWs used in the study by Moreira *et al.* (1994) and in the present study were derived from different European aquifers. Each MW source has a unique bacterial population profile (Guillot and Leclerc 1993), so bacteria present in one aquifer producing antagonistic and/or stimulatory substances may be absent from others. The above conflicting reports are derived using water from different sources, which will have different compositions and are likely to induce different autochthonous flora/pathogenic interactions.

In the present study, higher estimations of the concentration of the autochthonous flora were obtained from plate counts incubated at 22 °C than at 37 °C. This is in line with a study by Reasoner and Geldrich (1985) who reported that higher temperatures, i.e. 35–37 °C, were less suitable for estimating the autochthonous bacterial count in MW. This pattern of lower counts from plates incubated at higher temperatures would be expected, given that temperatures within MW aquifers are generally low. For example, the MW used in this study emerged from its underground source at less than 12 °C. Thus, it is not unexpected that the majority of bacteria found in MWs are psychrotrophs (Schmidt-Lorenz 1976) and, therefore, that the autochthonous flora is better adapted to growth at lower temperatures (i.e. 22 °C).

On day 0 no autochthonous flora were recovered on R2A, but on day 7 1·9 $\log_{10}$ were enumerated. Since the MW had been stored at 2 °C prior to the experiment, the flora normally recovered at 37 °C (mesophiles) may have become stressed and this, coupled with the fact that the lowest level of detection possible with the method used was $\geqslant 5$ cfu ml^{-1}, may explain why no autochthonous flora were detected on day 0.

At 22 °C, 2·5 $\log_{10}$ autochthonous flora were recovered from the MW on day 0, indicating that not all the bacteria recovered at this temperature had been stressed during storage at 2 °C. These bacteria may represent the psychrotrophic population present in the autochthonous flora. Irrespective of the incubation temperatures a dramatic increase in the number of autochthonous flora recovered was observed between 0 and 14 d at both 22 and 37 °C. The opening of the bottles, inoculating and dispensing into storage containers may have enriched the water with oxygen. These factors, combined with the upshift in storage temperature from 2 to 15 °C, may have contributed to the increase in aerobic autochthonous flora counts observed over the initial 14-d period.

The TEM studies of *E. coli* O157:H7 in MW demonstrated that, during extended storage, some *E. coli* O157:H7 cells

lyse, releasing their contents into the surrounding environment. The autochthonous flora may have been able to utilize some of these lysis products to assist in their survival. When the levels of the autochthonous flora recovered from inoculated (10^3 ml^{-1}) and uninoculated MW samples were compared from day 14 onwards, higher levels of the autochthonous flora were recovered from the inoculated MW samples compared with uninoculated samples. While this difference was not statistically significant, it highlights the fact that trace amounts of organic material due to the breakdown of *E. coli* O157:H7 cells may be capable of being used by the autochthonous flora for growth. In turn, an increase in the autochthonous flora concentration may allow extended survival of *E. coli* O157:H7, as demonstrated in this study at the 10^3 ml^{-1} inoculum level.

In conclusion, farm animals, in particular bovines, are the major reservoir for *E. coli* O157:H7. Thus the spreading of slurry onto farmland may act as a vehicle for transmission of the pathogen, because during wet weather slurry run-off may enter groundwater supplies.

Disturbance of the water table due to the exceeding of permitted extraction volumes of natural MW from aquifers may lead to increased opportunities for contaminated groundwater to gain entry into MW aquifers, resulting in contamination of natural MW intended for bottling. This present study confirmed that *E. coli* O157:H7 can survive for long periods in the nutrient-starved conditions offered by bottled MWs. The findings of this study, combined with the low infective dose of less than 50 cells, highlight the importance of the MW industries having adequate control measures in place to exclude this organism from their products.

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