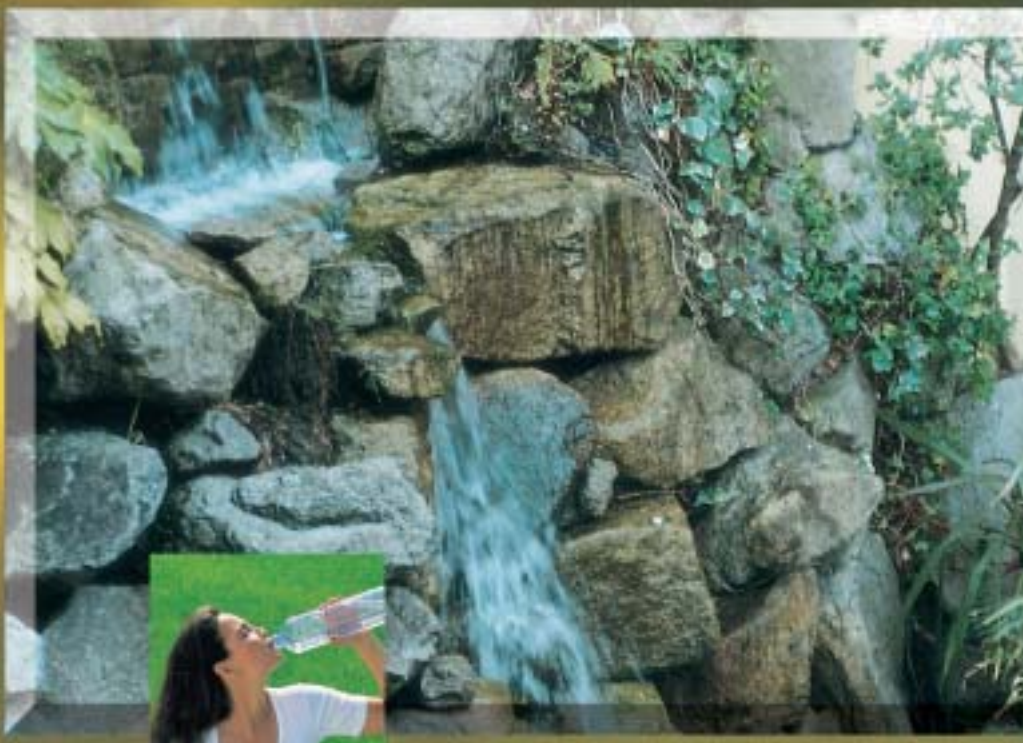
Agriculture and Food Development Authority

FINAL REPORT

Project Armis No.4392

The Survival of Added *Escherichia coli* O157:H7 in Natural Mineral Water



The National Food Centre

RESEARCH & TRAINING FOR THE FOOD INDUSTRY

RESEARCH REPORT NO 30

Contents

THE SURVIVAL OF ADDED *ESCHERICHIA COLI* O157:H7 IN NATURAL MINERAL WATER AND ITS PRODUCTS AND THE DEVELOPMENT OF A RAPID METHOD FOR ENUMERATION OF THE HETEROTROPHIC BACTERIA IN NATURAL MINERAL WATER.

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INTRODUCTION

The consumption of natural mineral water is rapidly growing and outpacing all other beverages on a global scale. In Europe, bottled water already has a bigger market share than carbonated soft drinks. Yet there is only a limited availability of information on the microbiological safety and quality of bottled natural mineral waters sold within the European Community. As natural mineral water does not receive any bacteriocidal treatment prior to bottling, the risk of pathogen contamination is a public health concern. Pathogen contamination may occur as a result of over exploitation of natural mineral water resources i.e. over abstraction by commercial bottling companies may lead to disturbance of the water table causing contaminated surface water to be drawn down into ground water supplies (Green and Green 1994). Such contamination was implicated in an outbreak of cholera associated with the consumption of bottled natural mineral water in Portugal in 1974 (Blake *et al.* 1977). The transport and dissemination of *E. coli* and enterococci in a limestone aquifer had been demonstrated by Personné *et al.* (1998), confirmation that *E. coli* can survive the transitory period from the surface to underground water supplies, thus raising the question of *E. coli* O157:H7 with its low infective dose < 10 cells (Willshaw *et al.* 1994 and Tilden *et al.* 1996) surviving the transitory period from surface to a natural mineral water aquifer.

Recognition of such risks has led to the introduction of regulations for acceptable microbiological quality and safety standards in EU Directive 80/777/EEC. This has led to regulation for controlled abstraction of ground water for commercial bottling in some, but not all, EU states. The development of rapid methods to assess the safety and quality of bottled natural mineral water is needed. Current standard methods for determining the heterotrophic plate count (HPC) take 3 days at 20 to 22 °C. Currently available rapid methods are largely unsuitable for enumeration of HPC bacteria in bottled natural mineral water. Their unsuitability is due to the physiological state of the naturally-occurring bacteria [autochthonous flora] present in natural mineral waters, which are often in a metabolically stressed or injured state. The delay in obtaining microbiological quality control results

delays the release of product onto the market and may lead to further financial losses in the case of natural mineral water being found to be outside specification.

This study was carried out in two parts, the objectives of which were:

- To determine the survival of *E. coli* O157:H7 in natural mineral water, as no previous studies had investigated the survival characteristics of this pathogen in natural mineral water (Part 1).
- To develop rapid methods for the enumeration of viable, heterotrophic bacteria in bottled natural mineral water, by using fluorochromes and direct epifluoresent microscopy (Part 2).

PART 1: THE SURVIVAL CHARACTERISTICS OF *E. COLI* O157:H7 IN BOTTLED NATURAL MINERAL WATER

The autochthonous flora offer a protective effect against the survival of *E. coli* in natural mineral water (Ducluzeau *et al.* 1976; Ducluzeau *et al.* 1984 and Lucas and Ducluzeau, 1990). However, no study has investigated the survival of the emerging pathogen *E. coli* O157:H7 in natural mineral water. This work investigated the survival of high concentrations of *E. coli* O157:H7 in natural mineral water, which could occur if slurry contaminated with *E. coli* O157:H7 leaked into an underground aquifer used for commercial bottling.

Method

Escherichia coli O157:H7 was inoculated to give a final concentration of 1000 cells per millilitre (3.0 $\log_{10}$ cfu per ml) into three water types, commercially-bottled natural mineral water, commercially-bottled natural mineral water from which the autochthonous flora had been removed by means of filtration, and sterile, distilled, deionised tap water which was free from both microbiological flora and minerals. Samples were examined every seven days to enumerate *E. coli* O157:H7 and the naturally occurring autochthonous flora.

Results

E. coli O157:H7 survived significantly longer in the natural mineral water than in the filter sterilised natural mineral water or the sterile, distilled, deionised tap water (Figure 1). Previous studies carried out by Ducluzeau *et al.* (1976), Ducluzeau *et al.* (1984) and Lucas and Ducluzeau (1990) had shown that the presence of the autochthonous flora had an antagonistic effect against invading pathogens such as *E. coli*. The presence of the autochthonous flora in natural mineral water in the present study did not have an antagonistic effect on the survival of *E. coli* O157:H7, in fact the presence of the autochthonous flora significantly extended the survival of *E. coli* O157:H7.

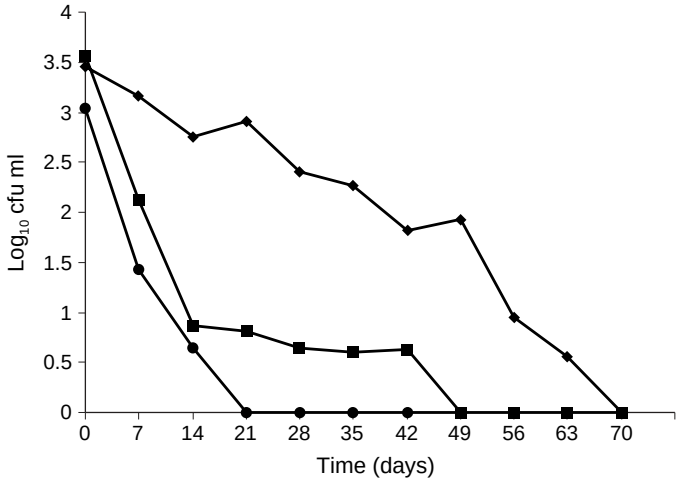


Figure 1: Survival of *Escherichia coli* O157:H7 in natural mineral water [◆], filter sterilised natural mineral water [■] and sterile, distilled, deionised tap water [●]. *Escherichia coli* was inoculated to give a starting concentration of 1000 cells per millilitre of each water type on day 0. *(cfu: colony forming unit.)*

Key to logarithmic scale (cfu per ml)	Real numbers (cfu per ml)
<0	<1 cell
0	1
1	10
2	100
3	1000
4	10000

SIMULATING THE SURVIVAL OF ADDED *E. COLI* O157:H7 IN COMMERCIALLY BOTTLED WATER

The prolonged survival of *E. coli* O157:H7 in the previous study raised the possibility of *E. coli* O157:H7 surviving in the commercial situation. To determine the survival characteristics of *E. coli* O157:H7 in commercially saleable bottles of natural mineral water, the pathogen was inoculated into the three water types to give low concentrations of 10 or 1 cell per millilitre of water type.

Method

E. coli O157:H7 was inoculated into unopened bottles of natural mineral water, sterile natural mineral water and sterile, distilled, deionised tap water to give final concentrations of 10 or 1 cell per millilitre of water type.

Results

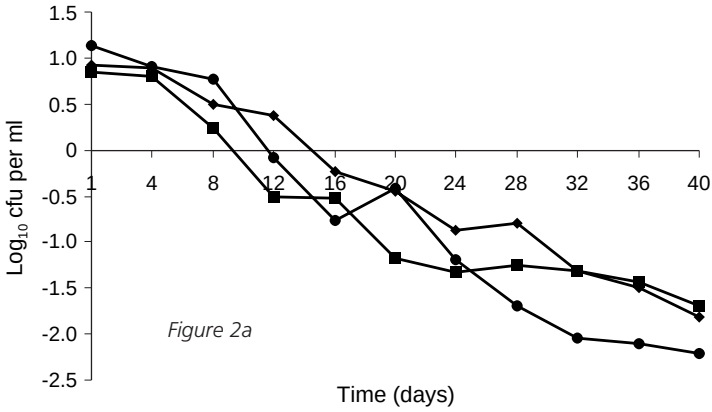
E. coli O157:H7 numbers survived as well or better in natural mineral water than in filtered natural mineral water or sterile, distilled, deionised tap water (Figures 2a and 2b). At a starting concentration of either 10 or 1 cell per millilitre, 1 *E. coli* O157:H7 cell per 160 ml of water was still detectable in the natural mineral water after 40 days storage. This is equivalent to 6 cells of surviving *E. coli* O157:H7 per liter of mineral water, sufficient to cause infection.

THE SURVIVAL OF ADDED *E. COLI* O157:H7 IN CARBONATED AND FLAVOURED NATURAL MINERAL WATERS

The survival of *E. coli* O157:H7 in low pH products, such as apple juice (Besser *et al.* 1993) and yogurt (Upton and Coia 1994) raises the possibility of its survival in carbonated (pH 5.0) and flavoured carbonated and non-carbonated natural mineral waters (pH 3.12) especially if an acid adapted strain contaminated these products.

Method

E. coli O157:H7 was inoculated into unopened bottles of non-carbonated and carbonated natural mineral waters, and into flavoured carbonated and non-



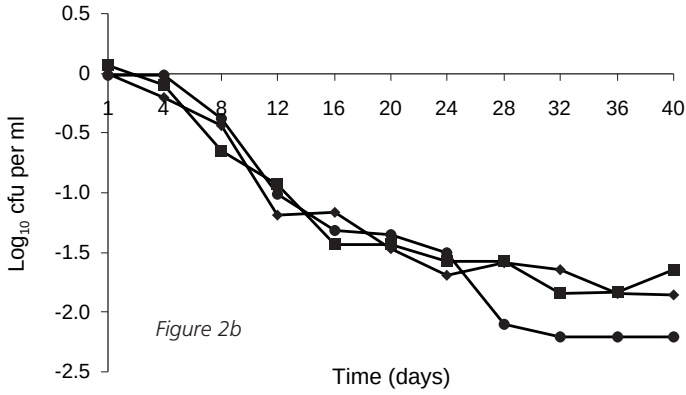


Figure 2: Survival of *Escherichia coli* O157:H7 in natural mineral water [◆], filter-sterilised natural mineral water [■] and sterile, distilled, deionised tap water [●]. The starting concentration of *Escherichia coli* O157:H7 was 10 cells per millilitre of water in Figure 2a and 1 cell per millilitre of water in Figure 2b.

carbonated natural mineral waters, to give final concentrations of 1000 cells per millilitre of water products.

Although flavoured natural mineral waters have preservatives present which are effective against a wide range of pathogens, Zhao *et al.* (1993) demonstrated the ability of *E. coli* O157:H7 to survive in apple cider in the presence of 0.1% potassium sorbate.

Results

The preservatives in the flavoured natural mineral water products had a significant effect on the survival of *E. coli* O157:H7 (Figure 3). The numbers of surviving *E. coli* O157:H7 in the flavoured natural mineral waters, containing sorbic acid at 250 parts per million and benzoic acid at 150 parts per million were reduced to less than 1 cell per ml within 4 days of inoculation. The numbers of surviving *E. coli* O157:H7 in the non-flavoured natural mineral waters i.e. carbonated and non-carbonated natural mineral waters (with a starting concentration of 1000 per millilitre of water) with no preservatives present were still detectable at 600 and 100 cells per millilitre respectively after 20 days of storage.

The study also compared the survival of a non-acid adapted strain of *E. coli* O157:H7 at a concentration of 10 cells with the same strain acid adapted in natural mineral water. No significant differences were found between the non-acid adapted and the same strain acid adapted indicating the natural resistance of *E. coli* O157:H7 to lower pH environments.

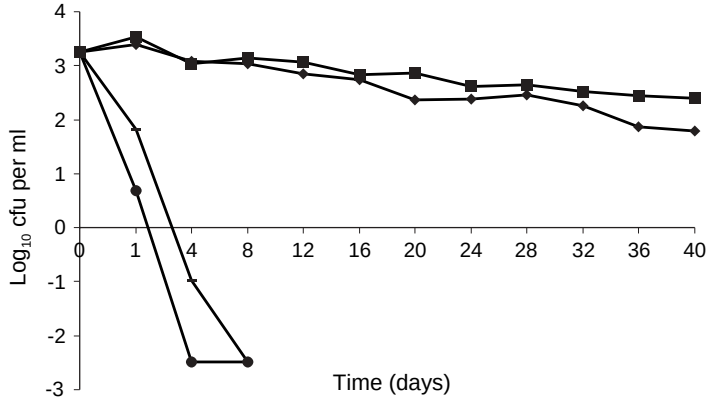


Figure 3: Survival of *Escherichia coli* O157:H7 in non-carbonated mineral water [◆], carbonated natural mineral water [■] flavoured non-carbonated natural mineral water [●] and carbonated flavoured natural mineral water [–]. The starting concentration of *Escherichia coli* O157:H7 on day 0 was 1000 cells per millilitre of water products.

CONCLUSION

The presence of the autochthonous flora in natural mineral water extended the survival of *E. coli* O157:H7. *E. coli* O157:H7 survived for several weeks in the extreme nutrient deprived conditions of natural mineral water. This is contrary to the findings of previous studies, which showed reduced survival in the presence of the autochthonous flora. The presence of preservatives in the flavoured natural mineral waters had a significant effect in reducing the survival of *E. coli* O157:H7.

RECOMMENDATIONS TO INDUSTRY

Since natural mineral water does not receive any bacteriocidal treatment prior to bottling, protection of the source is of the utmost importance. The protection afforded to natural mineral water sources warrant the strict regulations for registration of a natural mineral water company.

As cattle are assumed to be the main reservoir of *E. coli* O157:H7 this pathogen may easily find its way onto farmland in spring and early summer with the spreading of slurry. Personné *et al.* (1998) confirmed that *E. coli* on land can gain entry to ground water supplies. To date, investigations on the survival characteristics of *E. coli* O157:H7 indicate that this emerging pathogen is able to survive longer in the environment than other *E. coli* strains. Commercial water bottling companies therefore need to be vigilant and take precautions against the risk of this pathogen contaminating natural mineral water sources. Areas where control measures can reduce the risk are as follows:

- It is important that commercial mineral water companies do not over exploit resources by exceeding abstraction volumes during peak periods of demand i.e. summer season. The over abstraction of natural mineral water ultimately leads to disturbance of the water table and surface water being drawn down into ground water supplies.

- Any staff member recovering from an *E. coli* O157:H7 infection should remain at home until she/he has confirmation from their doctor of two consecutive stools that test negative for *E. coli* O157:H7.
- Products made from natural mineral water, such as the range of flavoured drinks contain nutrients capable of supporting the growth of contaminating pathogens, should have preservatives added. These preservatives should be investigated for their bacteriocidal activity against *E. coli* O157:H7 to ensure their effectiveness. Carbonated drinks often depend on the bacteriocidal activity of a lower pH. The current study indicates that lowering the pH may not be effective against *E. coli* O157:H7.
- In the event of *E. coli* O157:H7 contaminating bottled natural mineral water products, quality control procedures must be in place to alert staff and stop production. This will ensure the safety and quality of bottled water products to the consumer.

PART 2: RAPID METHODS FOR MEASURING THE BACTERIAL COUNT IN NATURAL MINERAL WATER

Total viable counts

The methods developed are outlined in Figures 4a and 4b. The methods used membrane filtration to concentrate the bacterial cells in a specific volume of natural mineral water onto a 0.2 µm polycarbonate membrane. The membrane was then stained using either the fluorochrome BacLight (BL) or the fluorochrome 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC). The stained membranes were visually assessed using an epifluorescent microscope with a 100 W mercury vapor light source and a 100 X oil immersion objective lens. Viable bacterial cells stained green when using the fluorochrome BL and red when using the fluorochrome CTC. The numbers of viable cells per ml

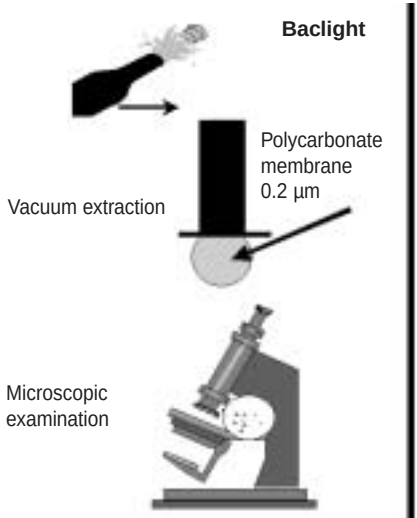


Figure 4a

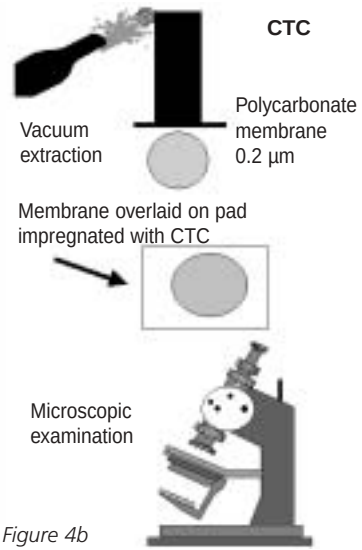


Figure 4b

Figure 4: Outline of the rapid method developed to measure the total viable bacterial count in natural mineral water. Method using the fluorochrome BacLight (BL) (Figure 4a) and fluorochrome 5-cyano-2,3-di-4-tolyl tetrazolium chloride (CTC) (Figure 4b).

were determined by counting the number of green fluorescent cells (BL) or red cells (CTC) in twenty random fields of vision.

Standard heterotrophic plate count

Standard heterotrophic plate counts (HPCs) were also carried out on the natural mineral water samples to enumerate the culturable counts, using either membrane filtration or the spread plate technique. The plates were then incubated at 22 °C for 3 days

The development of standard curves

The rapid direct counts for BL were plotted against the equivalent HPCs and a standard curve was derived with an equation for predicting the standard HPC at 22 °C for 3 days.

The rapid direct counts for CTC were plotted against the equivalent HPC after incubation at 22 °C for 3 days and a standard curve was derived with an equation for predicting the standard HPC at 22 °C for 3 days.

Validation

The developed methods using the viability staining procedures (BL and CTC) were applied and validated using a random selection of natural mineral water samples.

Results and discussion

Validation showed a linear relationship was obtained between the actual plate count at 22 °C and the counts predicted by BL. The correlation between the two methods was $r^2 = 0.86 \pm 0.5$ (Figure 5a). A linear relationship was also obtained between the actual plate count at 22 °C and the counts predicted by CTC. The correlation between the two methods was $r^2 = 0.82 \pm 0.62$ (Figure 5b).

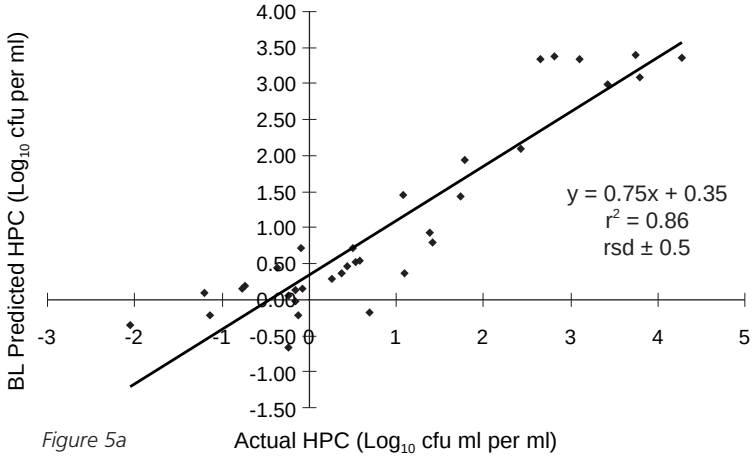


Figure 5a

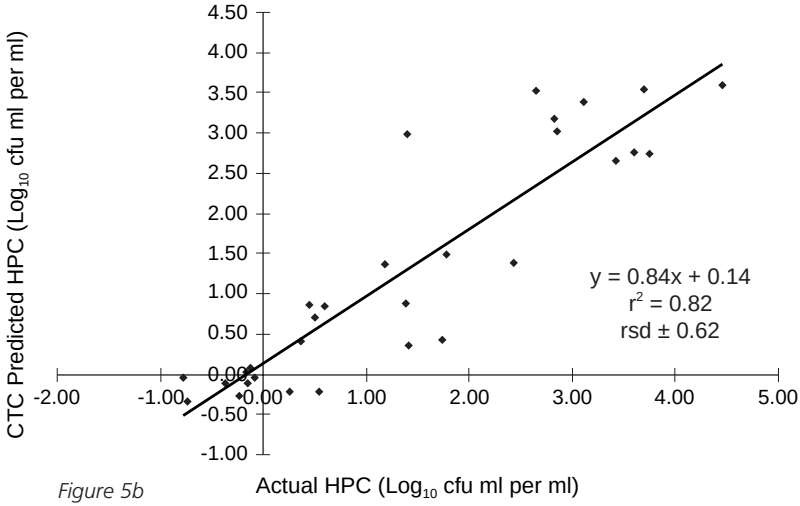


Figure 5b

Figure 5: Validation curves depicting the relationship between the BacLight (BL) direct count and the heterotrophic plate count (HPC) for bacteria incubated at 22 °C for 3 days on R2A agar (Figure 5a) and the relationship between the 5-cyano-2, 3-di-4-tolyl-tetrazolium chloride (CTC) direct count and the heterotrophic plate count for bacteria incubated at 22 °C for 3 days on R2A agar (Figure 5b).

CONCLUSION

These results indicate that the BL or CTC prediction equations can be used to predict the HPC equivalent to 22 °C for 3 days. Both fluorochromes are suitable for predicting the HPC present in bottled natural mineral waters. The BL method takes approximately 20 mins to complete while the CTC method takes approximately 2 hours.

RECOMMENDATIONS TO INDUSTRY

The current method for determining the HPCs takes 3 days at 20 to 22 °C. The delays in obtaining microbiological quality control results prevents rapid release of product into the market. The rapid methods developed can be used to predict the HPCs within 2 hours compared to the traditional standard method. Both new methods are simple, cost-effective and are suitable for use in an onsite laboratory. Flexibility of these techniques may be further improved when image analysis systems are developed which allow enumeration of the fluorochromes BL and CTC.

LIST OF PUBLICATIONS FROM THIS PROJECT

Kerr, M., Fitzgerald, M. Sheridan J.J., Mc Dowell, D. and Blair, I. (1999) Survival of *Escherichia coli* O157:H7 in bottled natural mineral water. *Journal of Applied Microbiology* **87: 833-841**

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Fitzgerald, M., Kerr, M., Sheridan J.J., Mc Dowell, D. and Blair, I. The autochthonous flora of natural mineral water: Changes in morphology and culturability following bottling. *Journal of Applied Microbiology* (submitted)

Fitzgerald, M., Kerr, M., Sheridan J.J., Mc Dowell, D. and Blair, I. The survival of of *Escherichia coli* O157:H7 in mineral water from different European Aquifers. *Journal of Applied Microbiology* (submitted)

Kerr, M., Fitzgerald, M. Sheridan J.J., Mc Dowell, D. and Blair, I.. The survival of *Escherichia coli* O157:H7 in still, carbonated and flavoured natural mineral waters. *Journal of Food Microbiology* (submitted)

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