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Survival and metabolic characteristics of Lux-Marked *Escherichia coli* O157:H7 in different types of milk

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Abstract

Escherichia coli O157:H7 is a potentially lethal pathogen which has been responsible for several outbreaks of milk-borne illness in recent years. The objective of this study was to evaluate the survival and metabolic activity (indexed by bioluminescence) of a chromosomally *lux*-marked strain of *E. coli* O157:H7 in raw, pasteurized and microfiltered pasteurized milk at 4 and 20°C for up to 14 d. Results showed that the population of *E. coli* O157:H7 and its metabolic activity decreased in all samples during storage at 4°C, with no significant differences in numbers observed between the different milk types; but the metabolic activity was significantly higher ($P<0.05$) in the microfiltered pasteurized milk than that in raw milk. At 20°C, *E. coli* O157:H7 counts and cell activity peaked at day 2, and then declined progressively. At 20°C, survival and metabolic activity were significantly lower in raw milk compared with pasteurized milk. We conclude that storage temperature is more important in regulating the survival of *E. coli* O157:H7 in contaminated milk than its origin/pre–treatment conditions.

***Keywords*:** Cross-contamination, dairy products, food poisoning, hygiene, microbiological quality.

Introduction

Due to the high nutrient content of milk, it is an optimal medium for the growth of several microorganisms (Barbano *et al*., 2006). Consumption of raw milk, if not heat–treated or pasteurized, can be particularly problematic and is responsible for many disease outbreaks worldwide. Outbreaks are also associated with improperly pasteurized milk, and dairy products made from unpasteurized milk (Vernozy–Rozand *et al*., 2005).

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Escherichia coli O157:H7 was first identified as a human pathogen in 1982 when outbreaks of bloody diarrhea and severe abdominal cramps occurred in the USA (Riley *et al*. 1983). The majority of affected individuals are children and the elderly, who can develop complications including haemorrhagic, diarrhoea, haemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Griffin, 1995). Though only a small percentage of raw milk samples have been found to be *E. coli* O157:H7 positive (Duncan and Hackney, 1994), contamination with this pathogen has resulted in several milk–borne outbreaks of gastroenteritis (Chapman *et al*., 1993). Since *E. coli* O157 is an ordinary inhabitant of the bovine intestinal tract, the route of contamination with *E. coli* O157:H7 is through faecal contact with feedstuffs, or during milking without strict hygiene practices (Hussein and Sakuma, 2005).

To date, the milk industry has successfully tackled issues of milk safety through various intervention strategies. Pasteurization has proved to be an effective measure in ensuring the safety of milk and dairy products. While unpasteurized raw milk can pose a public health concern, post–pasteurization contamination with *E. coli* O157 should also be noted. Faulty on–farm pasteurizers have also resulted in an outbreak of *E. coli* O157 (Goh *et al*., 2002). Incidentally, microbial growth has been shown to be greater in pasteurized samples of whey than its unpasteurized counterpart at a range of storage temperatures (Marek *et al*., 2004). Therefore, effective pasteurization and avoiding post–pasteurization cross–contamination in the fridge environment are both necessary to ensure the safety of milk and milk products (Heuvelink *et al*., 1998).

Although human pathogen outbreaks associated with milk are relatively rare, it is important to minimize this threat to maintain consumer confidence in dairy products and to protect the dairy industry. This study is the first to examine the metabolic activity of *E. coli* O157:H7 in different types of milk during storage, an important evaluator of the pathogen's potential infectivity (Jawhara and Mordon, 2004). The aim of this study was to improve our understanding of the pathogen's behaviour in milk through studying both its survival and metabolic activity in raw and different types of pasteurized milk under ambient (20 °C) and refrigeration (4 °C) conditions.

Materials and Methods

Preparation of milk

Raw milk was collected from the tank of a dairy farm located in Bangor, North Wales. The samples were kept at 4°C in sterile ice bags during transportation. Milk was used within 3 h after arrival at the laboratory. Part of the raw milk remained unpasteurized, whilst part was heat–treated in glass containers to 63.5°C (30 min) to prepare laboratory-pasteurized milk. Fresh full–fat commercially-pasteurized and full-fat microfiltered pasteurized milk (Cravendale) were purchased from Arla Foods UK Ltd (Leeds, UK).

Screening milk samples for *E. coli* O157:H7

Milk samples were tested for the presence of *E. coli* O157 before inoculation. Isolation and detection of *E. coli* O157:H7 involved enrichment followed by immunomagnetic separation (IMS). To start with, 5 ml of each milk samples were mixed with 45 ml of modified Tryptone Soy Broth (mTSB) (Oxoid CM 0989; Oxoid Ltd., Basingstoke, UK) and incubated at 37°C for 6 h. Afterwards, 1 ml of the enriched sample was analysed by Dynamag™–2 IMS (Invitrogen Dynal A.S., Oslo, Norway) with 0.02 ml of Captivate® *E. coli* O157 immunomagnetic beads (Lab M Ltd, Bury, UK) and incubated at 25°C for 30 min. After IMS, the beads were washed three times using phosphate buffered saline with 0.05% Tween 20 as wash buffer, and resuspended in 0.1 ml of the same buffer. They were then spread equally on three SMAC plates (sorbitol MacConkey agar plates (SMAC; Oxoid CM813) supplemented with cefixime (0.05 mg l^{-1}) and potassium telluride (2.5 mg l^{-1}) CT–SMAC), and incubated at 37°C for 18 to 24 h.

Inoculation of milk samples with *E. coli* O157:H7

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd, Teddington, Surrey, UK; 18 h, 37°C, 150 rev./min; Williams *et al*., 2008) of *E. coli* O157:H7 (Ritchie *et al*., 2003) in stationary growth phase. Cells were washed and concentrated by centrifugation as described in Avery *et al*. (2005). An inoculum (1 ml) of the mixture at the appropriate dilution was added to 99 ml of each milk type and mixed thoroughly in sterilised screw–cap bottles to obtain the desired final concentration of approximately 10^3 CFU ml^{-1}. All bottles of inoculated milk and uninoculated milk (control) were incubated at 4 and 20°C.

Survival and metabolic activity of *E. coli* O157:H7

E. coli O157 cells were enumerated at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d post–inoculation. Milk samples were serially diluted in Ringer solution (Oxoid), and serial dilutions were plated onto CT–SMAC and incubated at 37°C for 18 to 24 h. Non–sorbitol fermenting *E. coli* O157:H7 colonies were confirmed by agglutination with a latex test kit (Oxoid DR0620).

A parallel experiment was designed to assess variations in the activity of *E. coli* O157 among the different milk types (raw, laboratory–pasteurized, full-fat commercially-pasteurized, and microfiltered pasteurized). Bioluminescence of bacteria in milk was measured at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d post–inoculation. At each time–point, a 1–ml aliquot from samples used for the enumeration study detailed above was placed into a plastic luminometer cuvette and its luminescence (RLU) was determined using a SystemSURE plus Pi-102 Luminometer (Hygiena International Ltd, UK).

Aerobic plate counts and pH

Aerobic plate counts (APC) were determined from uninoculated milk samples (as control) at 0 (immediately after inoculation), 1, 2, 4, 6, 8, 10, 12 and 14 d. The uninoculated samples were serially diluted in Ringer solution, and serial dilutions (1:10) were plated onto plate count agar (PCA; Oxoid) and incubated at 30°C for 48 h. Samples' pH values were determined with a standard pH meter (Hanna instruments pH 211). Calibration was performed using two standard buffer solutions at pH 4.0 and 7.0.

Statistical analysis

Outcomes in the experiment were changes in *E. coli* O157:H7 cell counts and cell activity (bioluminescence), aerobic plate counts, and pH values during the 14 d incubation period. Log (y+1) transformation was performed on *E. coli* O157:H7 cell counts and cell activity, aerobic plate counts, which together with pH data were subjected to ANOVA tests and Tukey's test with significance at $p<0.05$ using SPSS 18.0 software (SSPS Inc, Chicago, Illinois, USA).

Results and Discussion

Screening milk samples for *E. coli* O157:H7

No *E. coli* O157:H7 was detected by the IMS method in any of the milk samples before inoculation.

Survival and metabolic activity of *E. coli* O157:H7

Survival and metabolic activity *of E. coli* O157:H7 at both 4 and 20 °C are shown in Figure (1). At 4 °C, *E. coli* O157:H7 populations declined steadily and continuously by 1.0-1.5 log10 CFU ml^{-1} in all samples over 14 d incubation. While log cell count reduction was greatest in raw milk (1.5 log10 CFU ml^{-1}), between-sample variations in survival of *E. coli* O157:H7 were not significant between all samples at this temperature ($p>0.05$). Metabolic activity of *E. coli* O157:H7 continuously and steadily reduced (by 1.3–2.07 log10 RLU) over the 14 d, with activity in raw milk diminishing near to zero. Cell activity in the microfiltered milk was significantly higher than that in raw milk ($p<0.05$), while no significant difference was seen among laboratory-pasteurized, commercially-pasteurized and raw milk ($p>0.05$). To our knowledge, Pasteurized and unpasteurized milk may be contaminated with *E. coli* O157:H7 when inadequate farm hygiene measures (milking and milk handling) are present or post-pasteurization contamination occurs. Given the low infective dose of *E. coli* O157:H7

(Chart, 2000) and the association of milk with past infections, it is important to understand the behavior of the organism in dairy products. Whilst others have previously studied changes in numbers of the organism in dairy products (Wang *et al*., 1997; Mamani *et al*., 2003; Marek *et al*., 2004). In addition, raw milk may also contain several compounds with bioactive components (e.g. lactoferrin, lactoperoxidase and lysozyme) that can reduce or eliminate populations of pathogenic bacteria; however, these will be lost during heat treatment (IDF, 1991). At 20 °C, *E. coli* O157:H7 cell counts in all milk samples showed a dramatic initial increase, peaking at day 2 (2.7-3.6 log10 CFU ml^{-1}), then progressively declined until the end of the 14 d incubation. Cell counts in raw milk samples decreased most (about 2.5-log cell count reduction using day 0 as baseline) and the count reduction was significantly higher (p<0.001) in raw milk compared with pasteurized samples. Counts did not statistically differ between the different types of pasteurized milk. Further ANOVA tests revealed that temperature was a significant factor moderating survival in all samples, with higher environmental temperatures leading to higher pathogen counts (p<0.001). Metabolic activity of *E. coli* O157:H7 at 20 °C in all milk samples increased significantly on day 1, which continued to rise and peak (2.3–2.75 log10 RLU) at day 2. Cell activity dropped significantly afterwards in all samples, reaching zero in raw milk at day 6 and at day 10 in pasteurized milk samples.

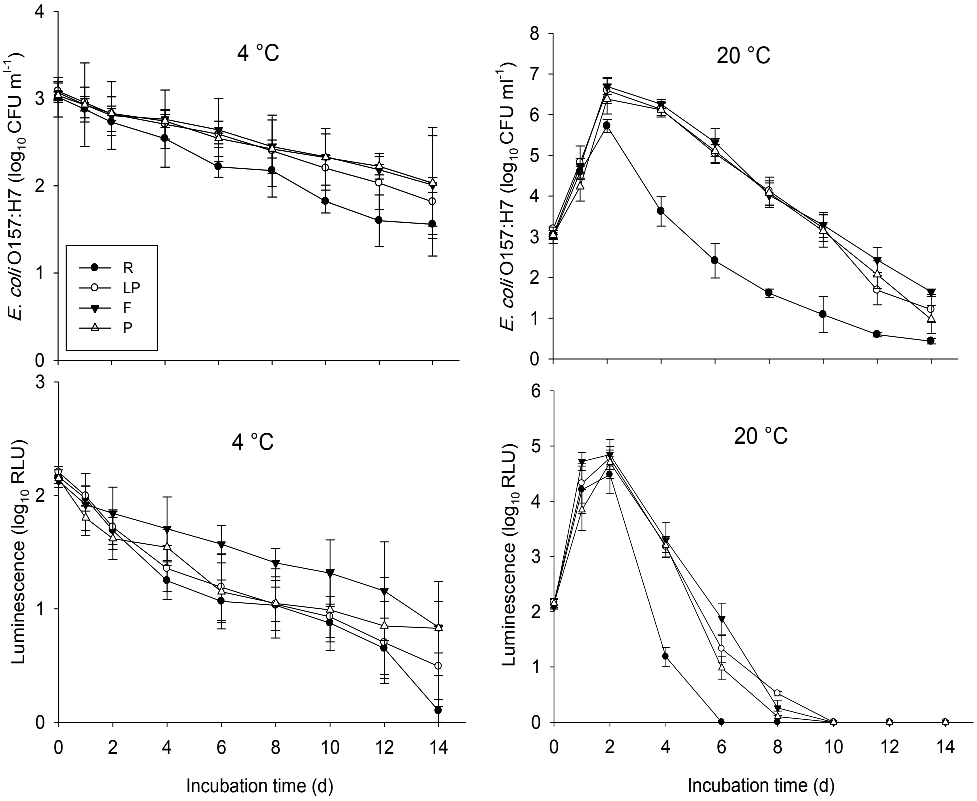

Figure 1. Survival and metabolic activity of *E. coli* O157:H7 in milk samples stored at 4 °C and 20 °C (R = raw milk, LP = laboratory–pasteurized milk, F = microfiltered pasteurized milk and P = commercially–pasteurized milk). Values represent means ± SEM (*n* = 3).

Cell activity in the three pasteurized milk samples was significantly higher than that in raw milk ($p<0.05$). Further ANOVA analysis indicated that temperature was an important influence on *E. coli* O157:H7 cell activity, with the higher temperature inducing a peak at day 2 which was not observed at the low temperature.

Aerobic plate counts and pH

Changes in APC (measured in log10 CFU ml^{-1}) and pH values are shown in Figure (2). At 4 °C, average APC increased significantly more in raw (around 3.9 log count growth) than in pasteurized milk (<1.35 log count growth) (all $p<0.001$, mean counts: raw>laboratory–pasteurized>commercially-pasteurized>microfiltered).

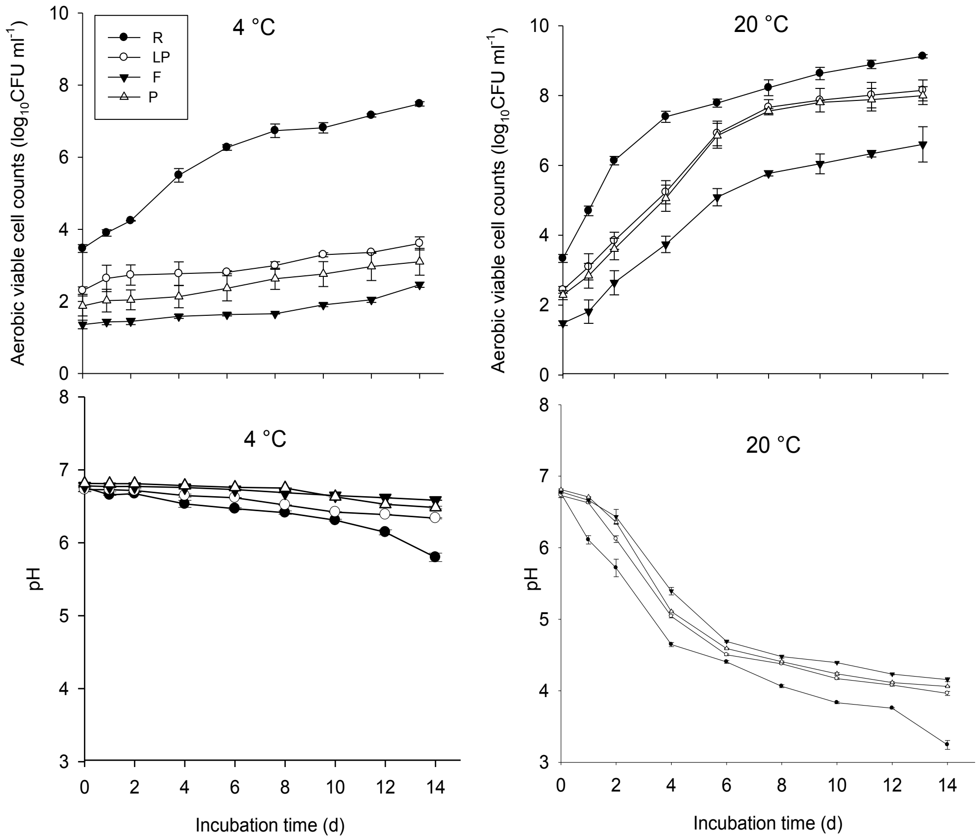

Figure. 2 Changes in aerobic plate counts (measured in log_{10} CFU ml^{-1}) and pH in milk samples stored at 4 °C and 20 °C (R = raw milk, LP = laboratory–pasteurized milk, F = microfiltered pasteurized milk and P = commercially–pasteurized milk). Values represent means ± SEM (n = 3).

At 20 °C, APC increased approximately 5–6 log10 CFU ml^{-1} in all milk samples after 14 d of incubation. APC in raw milk were significantly higher ($p<0.001$) than that in the three types of pasteurized milk, with values in microfiltered milk being significantly lower than those in the other pasteurized milk types ($p<0.001$). In the ANOVA test, temperature was found to be a significant factor in aerobic cell growth in the pasteurized milk samples. The growth rates in pasteurized samples were significantly higher under room temperature than under refrigeration temperature; although growth slowed down from day 8. In all, there was a final increase of around 6 log10 CFU ml^{-1} in APC from day 0 to 14.

At 4 °C, no substantial changes in pH were observed in the pasteurized milk samples (Figure 2), staying between 6.5 and 6.7; however, pH values in raw milk exhibited a gradual decrease to 5.7. Over 14 d at 20 °C, pH values decreased rapidly in all samples, from an average of 6.7 to 3.3 in raw milk, and to around 4.0–4.2 in pasteurized milk samples. Over the course of the experiment, there were significant differences in pH among the four types of milk (all $p<0.001$, pH mean: microfiltered>commercially-pasteurized>laboratory–pasteurized>raw). Changes in pH values were negatively associated with the increase in APC, with higher numbers of aerobic microorganisms leading to lower pH values.

The temperature is an important factor that influences the survival and activity of *E. coli* O157:H7. We observed that *E. coli* O157:H7 could not grow under refrigeration conditions in any type of milk, which was largely consistent with results from previous studies on a limited range of milk types (Wang *et al*., 1997). Previous studies have recommended that milk be kept at ≤5 °C as even at 7 °C, *E. coli* O157 can grow at a significant rate (Heuvelink *et al*., 1998). Whilst other studies have also found the organism to survive and proliferate at room temperatures (Wang *et al*., 1997; Mamani *et al*., 2003), this study additionally revealed a corresponding increase in the pathogen's metabolic activity at elevated temperatures.

E. coli O157:H7 numbers and metabolic activity consistently decreased at a greater rate in raw milk than in the three types of pasteurized milk. Greater APC values were recovered from raw milk and this is expected to result in elevated competition with, and/or antagonism against the pathogen, as reported elsewhere (Wang *et al*., 1997; Elwell and Barbano, 2006). Storage of raw milk at 20 °C also reduced pH considerably, most probably due to lactic acid production by the elevated counts of background micro–organisms (Kuippers *et al*., 2000). Acidic conditions (pH<3.5, Figure 2) are likely be detrimental to survival of *E. coli* O157:H7; however, it should be noted that the pathogen was not found to be eliminated at these low pH values, consistent with previous studies that show its acid resistance and adaptation in acidic environments (Leyer *et al*., 1995; Mamani *et al*., 2003; Carter *et al*., 2011).

Conclusion

The present study examining the role of incubation temperature has practical significance in understanding how *E. coli* O157:H7 and other aerobic cells behave in the food chain, from retailer fridge storage to the consumer home where the greatest risk of human infection occurs. We have shown that allowing contaminated milk to reach room temperature for even a space of 2 h can induce a transient proliferation of *E. coli* O157:H7 numbers and metabolic activity. Microfiltering milk did not have a significant effect on pathogen proliferation in comparison to normal pasteurization procedures and especially in comparison to the importance of storage temperate. Although pasteurization represents an effective measure to reduce pathogenic risks and improves the microbial quality of milk, consistent hygiene quality standards must be observed both pre- and post-pasteurization to guard against any possible pathogen and spoilage microorganisms.

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مدي بقاء والنشاط الايضي لبكتيريا *Escherichia* coli O157:H7 في أنواع مختلفة من الحليب

الملخص

تعتبر *Escherichia coli* O157:H7 من الميكروبات الممرضة والمسئولة عن تسجيل حالات وبائية عديدة في السنوات الاخيرة وذلك عن طريق انتقالها في الحليب. تهدف هذه الدراسة الي تقييم مدي البقاء والنشاط الأيضي لبكتيريا *Escherichia coli* ذات النوع المصلى O157:H7 في كل من الحليب الخام والمبستر والمصنع بطريقة البسترة والترشيح microfiltered، والذي تم تخزينهم لمدة 14 يوم على درجة حرارة 4 و 20 م°. أظهرت النتائج أن أعداد *E. coli* O157:H7 ونشاطها الأيضي قد أنخفض في جميع عينات الحليب التي تم تخزينها على درجة حرارة 4 م°، ولكن لم يكن هناك فروقات معنوية بين أعداد *E. coli* O157:H7 في تلك العينات، ووجد أن النشاط الأيضي لبكتيريا *E. coli* O157:H7 كان أعلى ($p<0.05$) في الحليب االمصنع بطريقة البسترة والترشيح microfiltered مقارنه بالحليب الخام. بينت الدراسة أيضاً أن أعداد *E. coli* O157:H7 ونشاط الخلايا في عينات الحليب قد بلغ ذروته في اليوم الثاني وذلك على درجة حرارة 20 م°، ثم بدأت تنخفض تدريجياً. كان مدى البقاء والنشاط الأيضي لبكتيريا *E. coli* O157:H7 على درجة حرارة 20 م° أقل في الحليب الخام ($P<0.05$) مقارنة بالحليب المبستر. خلصت هذه الدراسة إلى أن تأثير درجة حرارة التخزين تعتبر أكثر أهمية في تنظيم مدي بقاء *E. coli* O157:H7 في الحليب الملوث مقارنة بنوع الحليب وظروف ما قبل المعاملات التصنيعية له.

مفتاح الكلمات: التلوث العرضي، منتجات الألبان، التسمم الغذائي، الشؤون الصحية، الجودة الميكروبية.