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Heat and lime-treatment as effective control methods for *E. coli* O157:H7 in organic wastes

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ABSTRACT

Land-application of abattoir wastes is economically appealing and may provide an effective means of closing the nutrient cycling loop. This practise is constrained, however, by legislation which necessitates pre-treatment to remove pathogenic micro-organisms prior to land-spreading. Here we investigated whether heat-treatment or lime addition could eliminate *Escherichia coli* O157:H7 from three contrasting abattoir wastes. We found that treatment at 60 °C for 10 min effectively eradicated the organism while treatment for the same length of time at 50 °C led to 2–4 log reductions, but not a complete kill. Temperatures of 72 °C induced waste solidification rendering its use impractical. The potential for re-growth in heat-treated and untreated wastes was also investigated. Survival was significantly greater in heat-treated wastes, although the difference was less than half a log unit in magnitude. This effect of heat-treatment on pathogen survival appeared to be ameliorated when wastes were mixed with soil. No viable *E. coli* O157:H7 cells were recovered from any waste after application of lime (CaO) at a rate of 10 g l^{-1}, even after enrichment. Our results indicate that pasteurisation-style or liming treatments may provide a suitable alternative method for reducing pathogen loads in abattoir wastes, so that they can be applied to land with minimal biological risk.

1. Introduction

Escherichia coli O157:H7 has been implicated in human intestinal illness for over 20 years (Chart, 2000), and there is no obvious decrease in the number of UK cases reported (Health Protection Agency, 2008). Symptoms can occasionally be severe, culminating in complications such as haemolytic uraemic syndrome or thrombocytopaenic purpura. Fatalities occur in 5% of cases (Chart, 2000).

Ruminants (cattle and sheep) are the main environmental reservoir for this organism, which is shed in their faeces (Ogden et al., 2005; Omisakin et al., 2003). Another potential route of introduction to farmland is the application to land of animal-derived wastes, such as abattoir wastes (Jones, 1999). While this practise provides an important opportunity for reuse and disposal of organic wastes, *E. coli* O157:H7 has been shown to survive and even grow within these matrices (Avery et al., 2005; Hepburn et al., 2002; Williams et al., 2007). Following the implementation of the Animal By-Products Regulations (1774/2002), it is now illegal to spread untreated ruminant blood or waste containing ruminant blood onto agricultural land in Europe; and such waste must now be rendered or incinerated (Anon, 2005). This has resulted in a huge financial burden for abattoirs and the red meat-processing industry in terms of waste disposal costs. Furthermore, it represents a loss of a valuable nutrient resource and a proven plant growth enhancer (Abbas and Choudhary, 1995; Petroczki, 2004).

Outside Europe, a range of processes are currently employed for treatment of abattoir-derived wastes, including management *via* wastewater treatment plants, rendering, composting, and land-application. Although some methods have proven effective in reducing the microbial load of wastes (Bicudo and Goyal, 2003; Mittal, 2006), they may pose limitations. For instance, discharge to a wastewater treatment plant or rendering have substantial cost implications, whilst composting requires sufficient space, capital investment and maintenance (Mittal, 2006). Land-application appears appealing from an economic and sustainability standpoint, but is constrained by legislation to remove pathogenic micro-organisms prior to spreading. A pasteurisation-style heat-treatment or lime application could potentially provide solutions for treatment of organic wastes prior to application to land.

Although studies show *E. coli* O157:H7 is not particularly heat-resistant (Juneja et al., 1997; Stringer et al., 2000), the physical and chemical characteristics of media have been shown to influence the heat-resistance of the bacterium (Kaur et al., 1998; Stringer et al., 2000). Thus, it is possible that the variation in composition of abattoir wastes (Avery et al., 2005) could influence survival of

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this organism during heat-treatment. Importantly, heat-treatment is likely to alter the intrinsic physico-chemical and biological nature of organic wastes which may render them more or less favourable for re-growth of the organism should cross-contamination occur before or after application to land. Lime-treatment has been implemented to kill a range of enteric pathogens, including *E. coli* O157:H7, in sewage (Bean et al., 2007; Boost and Poon, 1998) and animal wastes (Duffy, 2003; Maguire et al., 2006; Ruiz et al., 2008). However, no study to date has investigated the effect of lime application on *E. coli* O157:H7 within abattoir waste.

The aims of this study were: (1) to determine whether a brief period of heat-treatment similar to the pasteurisation process, or addition of lime would be sufficient to inactivate *E. coli* O157:H7 in three different abattoir wastes, and (2) to compare the re-growth potential of the organism in heat-treated and untreated wastes, both alone and when combined with soil.

2. Methods

2.1. Bacterial strain

An inoculum was prepared from a fresh overnight culture (LB broth; Difco Ltd., Teddington, Surrey, UK; 18 h, 37 °C, 150 rev min^{-1}) of an environmental isolate of *E. coli* O157:H7, #3704 (Campbell et al., 2001) in stationary growth phase. Although the strain has been proven to be non-toxigenic due to the absence of toxin activity and toxin genes (Campbell et al., 2001), such strains still reflect survival patterns of toxigenic strains (Kudva et al., 1998; Ritchie et al., 2003). Cells were washed and concentrated as described in Avery et al. (2005).

2.2. Organic waste samples

Wastes used in this experiment consisted of ovine blood (OB), an ovine waste mixture comprising of blood, floor washings and gut contents (OM) and a mixture of sewage sludge, bovine blood, slurry, and creamery waste collected from a local waste contractor (CM).

2.3. Waste characteristics

All waste characterisation was performed upon four replicate samples. Electrical conductivity (EC; Jenway 4010 EC meter) and pH (Orion 410A pH meter) were measured directly. Waste moisture content was determined by drying at 80 °C overnight. Total carbon and nitrogen in each waste were quantified using a CHN2000 elemental analyser (Leco Corp., St. Joseph, MI). For further analyses, liquid wastes were centrifuged at 12,000*g* for 5 min and the supernatant retained for chemical analysis. Phosphate in the supernatant solution was determined colorimetrically *via* a modification of the method described by Anderson and Ing (1993) using a VersaMax microplate reader (Molecular Devices Ltd., Wokingham, UK). Nitrate and ammonium concentrations were determined using a Skalar San$^+$ segmented-flow autoanalyser (Skalar Analytical, Breda, The Netherlands).

An estimate of background heterotrophic bacterial counts was undertaken by performing serial dilutions of each waste and plating in duplicate onto R2A Agar (Oxoid Ltd., Basingstoke, UK). Colonies were enumerated after incubation at 20 °C for 48 h. Background counts of *E. coli* O157:H7 within the unspiked wastes were determined by performing serial dilutions on each waste, which were then plated in duplicate onto sorbitol MacConkey agar (Oxoid) containing 0.05 mg l^{-1} cefixime and 2.5 mg l^{-1} tellurite (CT-SMAC) (CT supplement; Dynal Biotech, Bromborough, UK). Plates were then incubated at 37 °C for 18 h prior to counting colonies with the characteristic morphology of *E. coli* O157:H7. To check for low numbers of the pathogen, an enrichment process was utilised, where 5 ml of each sample was shaken (150 rev min^{-1}, 6 h, 37 °C) in 15 ml modified Tryptone Soya Broth (mTSB; Oxoid) then streaked onto CT-SMAC agar and incubated as above. Plates were then scored for presence or absence of *E. coli* O157:H7.

2.4. Validation of batch heating technique

To validate the waste heating method employed in this study, aliquots of each waste type and corresponding volumes of water (controls) ($n = 4$) were placed in water baths for 10 min at one of the following temperatures: 50, 60, and 72 °C ($n = 4$); then cooled rapidly to 7 °C in a further water bath. Repeated temperature measurements were taken from the centre of all aliquots over this period using a digital probe (CheckTemp-1, Hanna Instruments Ltd., Beds., UK).

2.5. Resistance of E. coli O157:H7 in abattoir waste to heating

Sixteen replicate aliquots of each waste were placed into sterile microcentrifuge tubes and spiked with *E. coli* O157:H7 inoculum to give a starting concentration of approximately 7.2×10^5 colony forming units (CFU) ml^{-1} waste. These were subsequently vortexed (30 s) and allowed to equilibrate at room temperature for approximately 30 min. Heating treatments were then imposed by placing four tubes of each spiked waste in water baths for 10 min at one of the following temperatures: 50, 60, and 72 °C. A further four tubes were retained at room temperature (20 °C) as controls. Thereafter, tubes were removed from the water baths, and cooled rapidly to 7 °C, as described previously. Wastes were then homogenised by mixing by hand and then vortexing; and *E. coli* O157:H7 cells within the wastes were quantified on CT-SMAC as described previously. After plating, the remainder of the waste aliquot was placed into enrichment broth, as described previously. Where *E. coli* O157:H7 was detectable only following enrichment, samples were assigned an arbitrary value equal to half of the detection limit of plate counts (i.e. 2.5 CFU ml^{-1}).

2.6. Potential for re-growth of E. coli O157:H7 in heat-treated waste

2.6.1. Survival in heat-treated vs. untreated wastes

To assess whether heat-treatment of wastes rendered them more or less favourable for subsequent re-establishment of *E. coli* O157:H7, the bacterium's survival was monitored in heat-treated *vs.* untreated wastes. Aliquots of each of the three wastes were heated at 60 °C for 10 min (as described previously). Microcosms were prepared by placing 5 ml of waste (heated or not) inside individual 31 ml sterile plastic bottles to give 24 replicates of each waste type/treatment combination.

2.6.2. Survival in heat-treated vs. untreated wastes following mixing with soil

A further set of microcosms containing a mixture of 5 g soil (Eutric cambisol of the 'Denbigh' series; Williams et al., 2007) and 500 µl of waste were prepared, again providing 24 replicate microcosms for each waste type/treatment combination. Filled microcosms were loosely capped to allow aeration and placed in an incubator at 10 °C overnight to equilibrate. Both sets of microcosms were inoculated with 100 µl washed *E. coli* O157:H7 cells, prepared as described previously and mixed into the soil/ abattoir waste media. Microcosms were then replaced in the incubator at 10 °C. Four replicate microcosms per waste type/treatment combination were harvested destructively at 0, 2, 6, 13, 22 and 27 days following inoculation. At each harvest, microcosms were vortexed and plate counts were performed on dilutions of the contents of