IMPACT OF SOIL TYPE, BIOLOGY AND TEMPERATURE ON THE SURVIVAL OF NON-TOXIGENIC *ESCHERICHIA COLI* O157

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ABSTRACT

The occurrence of microbial enteropathogens in the environment can represent a serious risk to human health. The fate of enteropathogens introduced into the soil environment is dependent on a wide range of complex interacting environmental factors. While the effect of abiotic factors on enteropathogen survival has been widely examined, the interaction of enteropathogens with the soil microbial community is poorly understood. This study investigated the effect of soil biology and soil type on the survival of a non-toxigenic strain of *Escherichia coli* O157 under different temperature regimes. Soil microcosms of two soil types, with and without an intact microbial community, were inoculated with the enteropathogen surrogate, and survival was determined over a 64-day period, encompassing a shift from cold to ambient temperatures. In both soil types bacterial numbers decreased in soil with an intact microflora, while in the absence of an intact community *E. coli* populations increased. This effect was temperature specific, with *E. coli* populations remaining stable at low temperature, regardless of treatment. Soil type was of importance in survival at both cold and ambient temperatures. This work highlights the significance of the soil microbial community in suppressing enteropathogens in soil, and of investigating die-off in a multi-factorial manner.

INTRODUCTION

The occurrence of microbial enteric pathogens in the environment can represent a serious risk to human health. While numerous sources of pathogenic microorganisms have been identified in the environment, agriculture is often implicated as a significant contributor (Vernozy-Rozand *et al.* 2002; Burton and Turner 2003; Duffy 2003). Enteropathogens, including pathogenic bacteria, viruses, protozoa and helminths, may be released in large numbers in faecal material, deposited either directly by grazing animals or indirectly by the landspreading of animal manures, slurries and soiled waters (Gerba and Smith 2005; Chadwick *et al.* 2008). Among the principal pathogens of concern from agricultural sources is Vero cytotoxigenic *Escherichia coli* (VTEC), a subset of the enterohaemorrhagic *E. coli* (EHEC) group, which is highly pathogenic, has a low infectious dose and can cause severe illness in humans (Willshaw *et al.* 1994; EFSA 2007). The majority of human illness is caused by the O157 serotype, which can induce bloody diarrhoea and haemolytic uraemic syndrome (HUS), potentially resulting in renal failure and haemolytic anaemia (Porter *et al.* 1997). Ruminant animals, particularly cattle, constitute the primary reservoir of VTEC among livestock (Chapman *et al.* 1997; Nicholson *et al.* 2005). Once introduced into the environment VTEC can be transmitted to new human and animal hosts through direct contact with contaminated faeces or by ingestion of faecally contaminated food and water.

Survival times and die-off rates of enteropathogens in soils are critical for assessment of the risk posed to human populations by agricultural activities, with natural decay of pathogenic microbes in soil helping to prevent further transmission of infectious disease (Rosen 2000; Lang *et al.* 2003). Soil environments which favour prolonged survival and/or growth of enteropathogens can act as reservoirs for the subsequent contamination of disease vectors such as crops or water. The fate of enteropathogens introduced into the soil environment is dependent on a wide range of complex interacting environmental factors. These include the physical, chemical and biological properties of the soil, such as pH, soil moisture status, predation, soil type, nutrient availability,

soil structure and oxygen status (Sjogren 1995; Ishii *et al.* 2007; Semenov *et al.* 2008). Although there is extensive literature examining the effect of abiotic factors on enteropathogen survival in soil, studies investigating the impact of biotic factors, and particularly the soil microbial community, on survival are not as common (Jiang *et al.* 2002; van Elsas *et al.* 2007; 2012), and therefore the interaction of enteropathogens with the indigenous microflora is poorly understood. Biotic interactions that may affect survival include antagonism from indigenous microorganisms, competition for resources, predation and occupation of niche space (van Elsas *et al.* 2002). These biotic interactions would likely be strongly affected by both soil type and temperature. The effect of soil type and soil community status on *E. coli* survival in the context of a shift in temperature has not been previously investigated.

The objective of this experiment was to determine the effect of soil biology on the survival of a non-toxigenic strain of *E. coli* O157 in two soil types under different temperature regimes. The fate of *E. coli* O157 was investigated in relation to a shift from cold to ambient temperatures. This was carried out to approximate springtime conditions when livestock manures are applied. It was hypothesised that: (1) the indigenous soil microbial community would reduce *E. coli* survival as the soil community is better adapted to compete more effectively for space and nutrients; (2) the effect of the indigenous soil community on *E. coli* persistence would be temperature and soil-type dependent, with greater survival at low temperatures when the native community is metabolically less active, and in soils with a higher clay content, which would provide the *E. coli* with protection from predation and potential nutrient sources.

MATERIALS AND METHODS

SOIL COLLECTION AND PREPARATION

Two contrasting soil types from the Bearsted and Evesham series, both classified as Cambisols according to the World Reference Base (WRB 2006), were used in this experiment: (i) Bearsted soil (52.0075N, 0.4352W), a typical brown earth, described as coarse loamy passing to sandstone, pH 6.07 and C:N ratio of 10.6; (ii) Evesham soil (52.0048N, 0.4324W) a typical calcareous pelosol, described as clayey passing to clay or soft mudstone, pH 6.6 and C:N ratio 13.9. Replicate microcosms consisting of 5-g aliquots of each soil were prepared in 30-ml sterile glass vials for *E. coli* persistence incubations. Soil samples were collected to a depth of 0.15m from grass field margins that had not received livestock manure, or been grazed, within the previous 10 years. Soil was sieved to 4mm, mixed, and the water-holding capacity (WHC) was determined for each soil type according to the method described by Franz *et al.* (2011). Moisture content was then adjusted so that soils exhibited similar cohesiveness to achieve standard friability between different soil types, by wetting-up or restricted slow drying on the bench as appropriate. Following adjustment, soil moisture was measured by oven-drying at 105°C for 24h, and expressed as a percentage of WHC. This was determined to be 25% and 44% of the WHC for the sand loam and clay loam soils, respectively. For each soil type, sterile and non-sterile microcosms were established, providing an extreme contrast of soil biological communities. Soil microcosms were sterilised by autoclaving twice over a 2-day period (121°C for 1h at 1 bar). The remaining non-sterile microcosms had an intact microbial community. For each soil type, two treatments (sterile and non-sterile) were tested in independent triplicate replicates on each of seven sampling dates. Uninoculated microcosms for each treatment were used as a background control.

INOCULATION AND SURVIVAL ANALYSIS

Soil microcosms were inoculated with 1×10^8 colony-forming units (CFU) of *E. coli* O157:H7 strain 3704. This is a non-toxigenic VTEC strain originally isolated from the environment (Campbell *et al.* 2001). The inoculum was prepared by streaking a loopful of glycerol stock culture onto a Sorbitol MacConkey agar plate and incubating at 37°C for 24h. A colony was then picked from the plate and inoculated into 100ml Luria–Bertani (LB) broth, which was incubated as previously on an orbital shaker at 120rpm. An aliquot of 100μl was then transferred to fresh LB broth, and incubated a second time. This final *E. coli* culture was then spun down and washed three times with ¼ strength Ringer's solution (Oxoid) prior to inoculation within a volume of 500μl. After inoculation microcosms were incubated at 4°C for the first six days of the experimental period, and at 18°C thereafter. This temperature regime was chosen to simulate livestock manure application during a cold period followed by a period of warming, which would be typical of spring conditions in Ireland. All microcosms were mixed gently by hand following inoculation. Soil microcosms were incubated for up to 64 days and were destructively sampled immediately after inoculation, and on days 2, 4, 8, 16, 32 and 64 of the experimental period. Microcosms

were monitored for evaporation by weighing, and sterile water was added when necessary to maintain moisture levels. The extraction efficiencies (based on time 0 sampling time) for these soils were determined to be 58% and 83% for the clay loam and sand loam, respectively. *Escherichia coli* concentrations in the microcosms were enumerated by adding 10ml sterile ¼ strength Ringer's solution, vortexing for 1min and oscillating on a rotary shaker at 150rpm for 15min. Microcosms were then vortexed a second time and allowed to stand for 5min. The supernatant was serially diluted using ¼ strength Ringer's solution and plated on MacConkey sorbitol agar amended with cefixime-tellurite (Oxoid) using the spread plate technique. Plates were incubated at 37°C for 24h and characteristic beige colonies of non-toxigenic *E. coli* O157 were counted (Avery *et al.* 2005).

STATISTICAL ANALYSIS

The interactions between temperature, treatment (sterile/non-sterile) and time were analysed as a 2 × 2 × 7 factorial design, with soil type as a blocking factor. The Mixed Procedure in SAS (V9.1) was used to determine the effect of soil type, treatment and time interactions on *E. coli* O157 survival. All assumptions of the analyses were met. Values obtained at 4°C and at 18°C were analysed separately, to investigate *E. coli* response to warming temperatures that may be encountered during spring land applications. Soil types were also analysed separately to determine the effect of treatment over time. Finally, this procedure was used to determine the effect of the interaction between treatment and incubation temperature on survival. Multiple comparison *post hoc* analyses were performed with the Tukey honestly significant difference test. Mean CFU values were also used in Statistica (Version 10) to calculate the death rate (k-value) for *E. coli* in non-sterile sand loam and clay loam during incubation at 18°C. The first order decay function [$y = a + b \star e^{-kt}$, where y represents the population at a given time (log CFU g^{-1} soil), a represents stability of the final population at the end of the experiment (log CFU g^{-1} soil), b was the difference in population between the beginning and end of the experiment (log CFU g^{-1} soil), k represents the rate of population decline ($days^{-1}$), t represents time (days)] was used. This function has been used previously to estimate pathogen decay rates (Mubiru *et al.* 2000; Oliver *et al.* 2006). This function did not fit the population growth observed in the sterile microcosms so a linear function was instead applied to these treatments.

RESULTS

The persistence of the *E. coli* O157 (strain 3704) within the two soil types in both sterile and non-sterile microcosms is shown in Fig. 1. Overall,

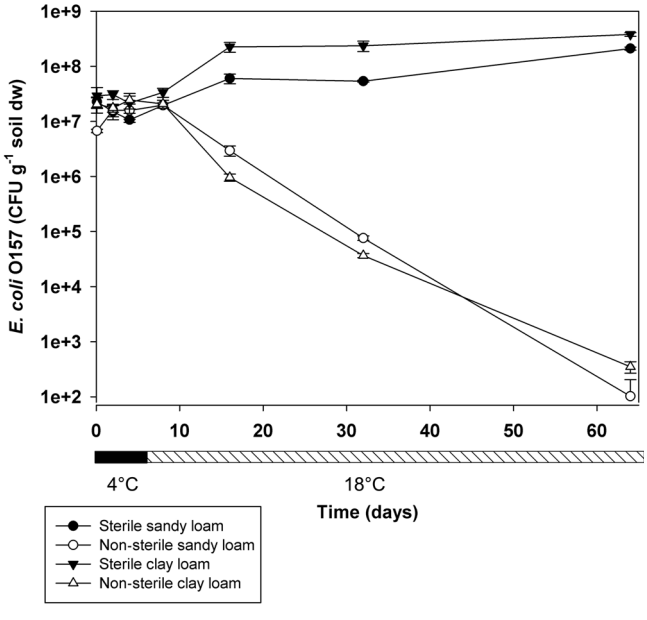

Fig. 1—Survival of *Escherichia coli* O157 in sterile and non-sterile sand loam and clay loam microcosms. Points denote mean CFU (n = 3); error bars denote standard error (note that some error bars fall within confines of symbols). Temperature is represented by the bar below the x-axis.

E. coli concentrations increased in sterile soil with the absence of an intact microbial community, while concentrations decreased in non-sterile soil. *Escherichia coli* concentrations were stable at 4°C, and while the *F* statistic (6.07) for soil type indicated a strong blocking effect at this temperature, there was no significant effect ($\alpha = 0.05$) of time, treatment, or their interaction with soil type on survival. When the incubation temperature was increased to 18°C, however a significant ($P < 0.0001$) three-way interaction was observed. In both soil types, a lag period was observed following the temperature change (day 6) before a difference between treatments became apparent, as there was no significant difference between sterile and non-sterile soils on day 8.

In sterile soil, *E. coli* populations increased in both sand loam and clay loam soils during the incubation at 18°C. This increase was significant initially ($P < 0.0001$); however, populations stabilised toward the end of the experiment. There was no significant difference in *E. coli* concentration in the clay loam soil between day 16 and 64 at 18°C. Similarly, there was no significant difference in *E. coli* concentration in the sand loam soil between day 16 and 32 at 18°C; however, *E. coli* increased significantly thereafter until the final sampling date on day 64 ($P < 0.0001$). Overall, there was a significant increase in *E. coli* concentrations in both soil types between day 8 and 64 at 18°C ($P < 0.0001$). In non-sterile soil, *E. coli* populations decreased in both soil types during incubation at 18°C. This decrease was maintained throughout, and was significant between each sampling interval for both soil types ($P < 0.0001$). There was also a significant effect of the treatment by temperature interaction on *E. coli* survival ($P < 0.0001$), but there was no difference in this effect between soil types. The interaction was significant for all combinations of treatments and temperatures with the exception of non-sterile and sterile soils during incubation at 4°C. Similarly, there was no significant effect of this interaction between sterile soils incubated at 4°C and 18°C.

Death rates were calculated for *E. coli* during incubation at 18°C and were found to be $0.24 day^{-1}$ and $0.36 day^{-1}$ in non-sterile sand loam and clay loam soils, respectively. Corresponding growth rates in sterile systems (calculated from the slope) were found to be $1.27 day^{-1}$ and $1.71 day^{-1}$ in sand loam and clay loam soils, respectively.

DISCUSSION

A fundamental effect of the indigenous microbial community on *E. coli* survival was strongly supported by the data presented here with the pathogen being found to grow in sterile soil in the absence of a microbial community, but die-off observed where the soil community remained intact. However, this treatment effect was temperature specific, being only observed at 18°C with *E. coli* concentrations remaining stable at low temperature, irrespective of soil biotic status. The observed decrease at 18°C was likely caused by enhanced activity of indigenous soil microorganisms and resultant antagonism toward the introduced pathogen. The effect of temperature on interactions between the pathogen and the soil community has been reported previously. Jiang *et al.* (2002) observed a more rapid decline of *E. coli* O157 in manure-amended unautoclaved soil at 21°C than at 5°C. This was attributed to an increase in microbial activity with temperature and consequently, greater competition for nutrients. Similarly, Vidovic *et al.* (2007) found that *E. coli* O157 declined more rapidly at 22°C compared to 4°C in unautoclaved soil. Predation of introduced *E. coli* by the soil community may have also played a role in the observed decline in non-sterile soil at 18°C. Sørensen *et al.* (1999) found that an introduced strain of *E. coli* K12 persisted in soil for 70d at 4°C and 10°C; however, it was no longer detectable after 20d at 25°C. The decline in *E. coli* K12 occurred with a corresponding increase in indigenous soil flagellates and ciliates. Similarly, Recorbet *et al.* (1992) noted that a decrease in the concentration of introduced *E. coli* coincided with an increase in the indigenous amoeba population.

An increase in *E. coli* concentration was observed in sterile soils in the absence of competitive and predatory interactions. The sterilisation process can alter the physical and chemical environment of the soil. Razavi and Lakzian (2007) found a significant increase in the concentration of extractable organic carbon in autoclaved soil as compared to a control using the chloroform fumigation extraction (CFE) technique. This increase was attributed to the breakdown of humic substances and the death of microorganisms. Also, Unc and Goss (2006) observed a significant increase in the number of *E. coli* in manure-amended sterile soil. This was because of not only the elimination of competitors and predators during sterilisation, but also the extra carbon released by cell lysis. In this case, the additional carbon may have served as a significant food source for the pathogen.

Soil type is another important aspect of pathogen survival, as different soils have unique biogeochemical, structural and textural properties that determine the availability of substrate, water and habitats. It was hypothesised that

greatest persistence and growth would be observed in non-sterile and sterile clay loam microcosms, respectively, compared to corresponding sand loam microcosms, and this was supported by the data presented here. Soil type was a significant factor affecting pathogen survival at 18°C in this study. Typically survival is positively associated with clay content because of inherent clay properties such as fine texture (Cools *et al.* 2001), micropore availability (Postma *et al.* 1990; Wright *et al.* 1995) and nutrient adsorption (Coleman *et al.* 2004). For example, Fenlon *et al.* (2000) could isolate exogenous *E. coli* for four months from clay and loam soils compared with eight weeks from sand soils. Additionally, Franz *et al.* (2008) showed a negative association between *E. coli* O157:H7 survival and number of clay particles in manure-amended loamy soils. However, contrary to the original hypothesis, this experiment found that *E. coli* decayed more rapidly in clay loam soil. Other work has shown that organic matter (OM) can be more influential than soil texture in determining pathogen survival (Cools *et al.* 2001; Lang and Smith 2007). Therefore, persistence in sand loam soil in this instance may have been because of the confounding effect of differences in soil OM and microbial community composition between soil types. Nonetheless, the greatest absolute numbers of *E. coli* were isolated from the clay loam soil at the end of the experimental period, thus confirming the importance of clay in long-term *E. coli* persistence, which has been previously described (Brennan *et al.* 2010). Additionally, the highest growth rate was achieved in clay loam soil. This was likely caused by greater nutrient availability associated with clay content, which may have facilitated a more rapid expansion of the *E. coli* population.

To conclude, *E. coli* introduced to cold soils through springtime application of livestock manure may form an environmental reservoir, persistent whilst such conditions prevail. However, based on these results, this reservoir may be depleted as temperatures increase if the native community is intact. However, if the native community has been compromised, this reservoir may potentially increase with increasing temperatures as the result of competition for nutrients and antagonistic interactions. This shows the importance of a healthy soil microbial community for effective pathogen suppression, and of investigating die-off in a multi-factorial manner.

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