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The Survival of *Salmonella* Senftenberg, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium sporogenes* in Sandy and Clay Loam Textured Soils When Applied in Bovine Slurry or Unpasteurised Digestate and the Run-Off Rate for a Test Bacterium, *Listeria innocua*, When Applied to Grass in Slurry and Digestate

Lauren Russell[1,2†], Paul Whyte[2], Annetta Zintl[2], Stephen V. Gordon[2], Bryan Markey[2], Theo de Waal[2], Stephen Nolan[3], Vincent O'Flaherty[3], Florence Abram[3], Karl Richards[4], Owen Fenton[4] and Declan Bolton[1†]*

[1] Teagasc Food Research Centre, Dublin, Ireland, [2] Herd Health Unit, School of Veterinary Medicine, University College Dublin, Dublin, Ireland, [3] Microbial Ecology Laboratory, School of Natural Sciences, National University of Ireland, Galway, Ireland, [4] Teagasc Environmental Research Centre, Wexford, Ireland

This study investigated the survival of *Salmonella* Senftenberg, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Enterococcus faecalis* and *Clostridium sporogenes* in sandy and clay loam textured soils when applied in bovine slurry or unpasteurised digestate, using laboratory based inoculation studies. The run-off rate for a test bacterium, *Listeria innocua*, when applied to grass in slurry and digestate, was also examined using field studies. Bovine slurry and digestate were inoculated with the target bacteria to a final concentration of 10^6 $\log_{10}$ cfu/g or spores/g, thoroughly mixed into soil samples and incubated at 4°C or 14°C. Samples were removed periodically and the surviving cells enumerated using AOAC or equivalent methods. The loss of viability/culturability phase followed first order kinetics and T_{90} values ranged from 11.9 to 166.7 d at 4°C and from 6.0 to 156 d at 14°C. With the exception of *E. coli* O157:H7 and *E. faecalis* in sandy loam textured soil at 14°C (T_{90} values were significantly ($P < 0.05$) higher in slurry) the type of soil texture or application material (slurry or digestate) did not affect survival rates. In the field study, 12 grass covered micro-plots were prepared. *L. innocua* was applied in digestate and bovine slurry and rainfall was simulated at a target rate of ∼11 mm per plot per h^{-1}. Rainfall simulation (30 min) took place after 24, 48 h, 14 d and 30 d. Run-off

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***Correspondence:**
Declan Bolton
declan.bolton@teagasc.ie

†These authors have contributed equally to this work and share first authorship

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samples were tested for the *L. innocua* strain using Brilliance *Listeria* agar supplemented with streptomycin sulphate (1,000 μg/ml) at 37°C for 48 h, as were soil samples after 30, 58, 86 and 112 d. Significantly ($P < 0.05$) lower counts were obtained in the run-off from digestate after 1, 2 and 30 d as compared to slurry. It was concluded that the type of organic fertiliser does not affect the bacterial survival rates in sandy and clay soils, with the exception of *E. coli* O157:H7 and *E. faecalis* in sandy loam textured soil at 14°C. Furthermore, bacteria may be retained better in the soil-digestate matrices during rainfall although additional research is required to further validate and provide the scientific basis for this observation.

Keywords: bacterial pathogens, survival, soil, slurry, digestate, runoff

INTRODUCTION

Anaerobic digestion (AD) is an efficient and environmentally sustainable method for processing large amounts of farm, food and wastewater treatment materials (Nolan et al., 2018). Increasing the application of AD is a key objective of the European Green Deal, the blueprint for turning the EU into the first climate neutral continent by 2050 (European Commission, 2020). Co-digestion of combined wastes produces biogas (methane) and digestate, a nutrient rich organic material frequently used as a fertiliser (Alkanok et al., 2014). In addition to contributing to international renewable energy targets, land spreading digestate recycles nutrients from biowaste back into food production, a critical activity in sustainable farming (Johansson et al., 2005). Historically, bovine slurry, which makes up ∼80% of the 1.6 billion tonne of animal waste produced in Europe every year (Foged et al., 2011), has been used as the main organic fertiliser on farmland. However, as the use of AD has increased, so too has the application of digestate.

Both slurry and digestate enhance soil health while providing organic matter and essential plant micronutrients (Nikoli and Matsi, 2011; Larkin, 2015; Bhattacharya et al., 2016; Slepetiene et al., 2020). Slurry is often contaminated with a range of bacterial, viral and parasitic pathogens including *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Streptococcus pyogenes*, enteropathogenic *E. coli* (EPEC), *Clostridium* spp., *Campylobacter* spp. *Mycobacterium* spp., *Cryptosporidium parvum, Giardia* sp., swine Hepatitis E, and norovirus (Alam and Zurek, 2006; Ferens and Hovde, 2011; Russell et al., 2020). Digestate may also contain pathogens from contaminated feedstock materials (Russell et al., 2020), that survive the AD process (Olsen and Larsen, 1987; Sahlström et al., 2004; Bagge et al., 2005; Goberna et al., 2009). Post-reactor contamination from the environment, insect and animal vectors, may also occur during storage (Sidhu et al., 2001; Bagge et al., 2005; Zaleski et al., 2005; Pepper et al., 2006). The absence of unpleasant odours, reduced methane emissions, decreased biochemical oxygen demand and a more balanced nutrient mix and bioavailability make digestate more suitable for land spreading than slurry. However, digestate has the added risks of introducing pathogens such as *E. coli* O157, *Salmonella, Listeria*, *Campylobacter*, *Cryptosporidium*, *Ascaris*, *Mycobacterium avium* subspecies *paratuberculosis*, and *Giardia*' not already present on the farm receiving the digestate and/or disseminating pathogens from a single source to multiple farms (Nolan et al., 2018).

Current European Regulations (EC) 1774/2002 and 1069/2009 permit the application of animal wastes and digestate as organic fertilisers and soil improvers and set limits for the pathogen loads permitted, eg. digestate must have faecal indicator bacteria (total coliforms, *E. coli*, and *Enterococcus* spp.) <1,000 cfu g^{-1} to be permitted for landspreading, while highlighting the requirement for further research on the risks associated with pathogens in slurry and digestate when applied to agricultural land. Crops contaminated with enteric pathogens from organic fertilisers, spread *via* water and soil have been a source of human illness (Warriner et al., 2009; Castro-Ibáñez et al., 2015). Indeed, in recent years fresh produce has surpassed meat as the leading source of foodborne illness outbreaks (Herman et al., 2015). Waste materials, temporarily mobilised during rainfall events may spread to adjacent fields or rivers, further disseminating pathogens like *Salmonella* spp. and *E. coli* O157 (Ongeng et al., 2015; Peyton et al., 2016; Clagnan et al., 2019). However, the opportunity to cross-contaminate crops is dependent on the survival properties of the bacteria and the environmental conditions. It is generally accepted that soil is a hostile environment where enteric bacteria die-off as a result of exposure to stresses such as suboptimal temperatures, low water availability, metal toxicity (especially copper and zinc), nutrient deprivation, exposure to metabolites from soil microorganisms and UV combined with predatory protozoa (Artz and Killham, 2002; Coohill and Sagripanti, 2009; Ongeng et al., 2015). Thus, soils are a dynamic environment with multiple and interacting factors influencing survival, which may also be affected by the application material (slurry or digestate) (Ongeng et al., 2015).

Previous research has investigated the survival of bacteria in soil amended with animal slurry including *Salmonella* in soils amended with pig manure (Holley et al., 2006) and bovine slurry (Nicholson et al., 2005) as well as *E. coli* O157:H7 and *Enterococci* in bovine faeces spread on grassland (Bolton et al., 1999; Byappanahalli and Fujioka, 2004). However, there is a dearth of similar studies for digestate. Moreover, to the best of our knowledge, studies that compare bacterial survival in soils amended with slurry vs. digestate are limited. Survival studies are therefore required to determine the survival of key pathogens

in slurry and digestate amended soils. Laboratory studies, where variables other than the application material can be controlled, are particularly useful for this but may not be representative of the dynamic nature of the natural environment especially the impact of rainfall on the physical removal of bacteria (Ongeng et al., 2015), whereas field-based trials are more representative (Wang et al., 2018). Moreover, such studies often used a rainfall simulator to examine bacterial run-off from soil under field conditions (Brennan et al., 2012; Healy et al., 2017).

The primary objective of this study was to investigate bacterial pathogen survival in amended soil when either slurry or digestate was applied to the two most common soil types in Ireland using laboratory trials. A second study was also undertaken using rainfall simulation in a field trial that investigated the run-off rate of *Listeria innocua* when applied in slurry and digestate.

MATERIALS AND METHODS

Laboratory-Scale Soil Survival Study

Soil Preparation

A sandy loam (52% sand, 30% silt, 18% clay) and a clay loam (44% sand, 36% silt, 20% clay) soil (Brennan et al., 2012) were extracted from a permanent grassland site in Teagasc Johnstown Castle, Co. Wexford, Ireland. Pebbles and small stones were removed by hand prior to portioning into samples. In total 40 boxes (GenBox Jar, 2.5L; Biomeriéux, Basingstoke, UK) were labelled, 20 as "sandy loam" and 20 as "clay loam", and 450 g of the appropriate soil sample was weighed into each. These boxes were sealed to prevent moisture loss and wrapped in aluminium foil to exclude light.

Inoculum Preparation

Marked strains (streptomycin resistance 1,000 μg/ml) of *Salmonella* Senftenberg and *Listeria. monocytogenes*, and reference strains of *E. coli* O157 (EDL 933), *Enterococcus faecalis* (NCTC 12697) and *Clostridium sporogenes* (DSM 767), used to represent proteolytic *C. botulinum* were obtained from the Teagasc culture collection held in the Teagasc Food Research Centre (Ashotwn, Dublin, Ireland). To prepare inocula for all target bacteria, except *C. sporogenes*, a cryoprotective bead from frozen (−80°C) storage was streaked on Tryptone Soya Agar (TSA; Oxoid, Fannin Ltd., Ireland) and incubated at 37°C for 24 h. A single colony from the resultant culture was aseptically mixed into 10 ml of Tryptone Soya Broth (TSB; Oxoid, Fannin Ltd., Ireland) and incubated overnight at 37°C. The culture obtained was centrifuged at 8,014 × *g* (Centrifuge 5801 R, Eppendorf, Cambridge, UK) for 10 min, washed and resuspended in phosphate buffered saline (PBS; Oxoid, Fannin Ltd., Ireland) three times, before resuspension in PBS and serially diluted to obtain cell concentrations of $\sim 10^7$ cfu/ml.

Dried *C. sporogenes* cultures obtained from DSMZ were rehydrated, following the instructions provided, in an anaerobic environment. *C. sporogenes* spores were prepared by first adding 100 μl of rehydrated culture to 20 tubes of cooked meat medium (CMM; Oxoid, Fannin Ltd., Ireland) broth (20 ml) and incubating in an anaerobic workstation (Don Whitley, United Kingdom) for 12–18 h at 37°C. *Clostridium* sporulation agar was prepared as described by Casadei et al. (2000) and placed in a Whitley A35 anaerobic chamber (Don Whitley Scientific, West Yorkshire, UK) overnight using the ANO_2 gas mixture (10% H_2, 10% CO_2, 80% N_2; Air Products Ireland, Dublin, Ireland) to exclude all oxygen. Aliquots (300 μl) of the overnight CMM broth were then spread onto 300 plates of *Clostridium* Sporulation Agar (CSA) (Casadei et al., 2000) (inside the anaerobic chamber) before transfer to anaerobic boxes (GenBOX jars; BioMérieux UK Ltd., Basingstoke UK) containing Anaerogen sachets (Oxoid, Fannin Ltd., Ireland) and incubated at 37°C for 12 days. Spores were harvested by adding ∼5 ml ice-cold sterile distilled water onto the surface of the CSA plates and agitating the surface of the agar with a sterile spreader to release spores from the agar. The suspension was then transferred to the next agar plate and the process repeated. The suspensions were then pooled in 50 ml tubes, centrifuged at 7,500 x g at 4°C for 10 min and washed with iced water, reducing the amount of liquid over the course of repeated cycles until a spore suspension of $\sim 10^7$ spores/ml (estimated by phase contrast microscopic examination) was obtained. The spore suspension was kept on ice throughout the entire harvesting process. This concentration was then confirmed by plating out on Columbia Blood Agar (CBA; Oxoid, Fannin Ltd., Ireland) with 5% defibrinated horse blood (Cruinn diagnostics, Ireland) in anaerobic conditions as described above. The spore preparations (1 ml aliquots) were stored at −80°C. Prior to inoculation, spore preparations were thawed at room temperature and heat treated at 80°C for 10 min to ensure the destruction of any vegetative cells.

Soil Inoculation and Storage

Fresh bovine slurry was obtained from a beef farm in County Meath, Ireland, while digestate was obtained from a commercial AD plant in the east of Ireland that operates a mesophilic, industrial scale continuous system, using mainly food waste and slurry as feedstock materials. Twenty slurry samples (45 g) and twenty digestate samples (45 g) were prepared. For each bacterium of interest, four digestate and four slurry samples were inoculated in 5 ml PBS to give a concentration of 10^6 $\log_{10}$ cfu/g or spores/g. These samples were then thoroughly mixed manually using a sterile metal spoon into the previously prepared soil samples (450 g) to obtain four of each of the following combinations for each target bacterium: digestate plus sandy loam, digestate plus clay loam, slurry plus sandy loam, and slurry plus clay loam. The boxes were sealed and 2 of each combination incubated at 4 or 14°C (mean winter and summer temperatures respectively in Ireland) and sampled at times (t) = 0, 12, 26, 40, 54, 68, 82, 96, 110 and 124 d. Immediately prior to sampling the contents were thoroughly mixed again and 10 g removed.

pH and Water Activity

The pH and water activity (a_w) of the soil samples was recorded at each sampling time-point. The pH was tested using a Eutech pH 150 probe (Thermo Scientific, USA) which was calibrated using pH 4, 7 and 10 standards prior to use. The water activity was measured using an Aqualab Pre water activity metre (Labcell). The metre was calibrated prior to use using a saturated solution of sodium chloride (KCl, $a_w = 0.984 \pm 0.003$ at 20°C).

Microbiological Analysis

Direct Counts

A 10 g soil sample was added to 90 ml of maximum recovery diluent (MRD, Oxoid, Fannin Ltd, Ireland) and stomached (Colworth Stomacher 400) for 60 s. A serial dilution was prepared in MRD and the target bacteria were enumerated in duplicate using selective agar as follows: (1) Xylose Lysine Desoxycholate agar (XLD: Oxoid, Fannin Ltd., Ireland) supplemented with streptomycin sulphate (1,000 μg/ml; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) incubated at 37°C for 24 h for *S.* Senftenberg; (2) Sorbital MacConkey Agar supplemented with cefixime-tellurite (CT-SMAC: Oxoid, Fannin Ltd., Ireland) at 37°C for 24 h for *E. coli* O157:H7; (3) Brilliance *Listeria* Agar (OCLA; Oxoid, Fannin Ltd., Ireland) with streptomycin sulphate (1,000 μg/ml) incubated at 37°C for 48 h for *L. monocytogenes*; (4) Slanetz and Bartley Agar (Oxoid, Fannin Ltd., Ireland) incubated at 37°C for 24 h, followed by 42°C for a further 24 h to enumerate *E. faecalis*; and (5) Reinforced Clostridial Agar (RCA; Oxoid, Fannin Ltd., Ireland) incubated anaerobically, using Anaerogen sachets in anaerobic jars at 37°C for 48 h for *C. sporogenes*. As RCA is not sufficiently selective, presumptive *C. sporogenes* colonies were confirmed using real-time PCR, as described by Morandi et al. (2015).

Enrichment

Enrichment procedures were used to detect target bacteria when the residual concentration was below the limit of detection using direct plating methods. Enrichment cultures of *S.* Senftenberg were prepared by adding 10 g of the soil sample to 90 ml buffered peptone water (BPW) and incubating at 37°C for 24 h before plating on Modified Semi-Solid Rappaport Vassiliadis medium (MSRV; Oxoid, Fanning Ltd., Ireland) with novobiocin supplement (20 mg/l, Oxoid, Fannin Ltd., Ireland) and incubating at 42°C for 24 h. Presumptive *Salmonella* colonies which exhibited haloed growth were streaked onto Xylose Lysine Deoxycholate agar (XLD, Oxoid, Fannin Ltd, Ireland) and incubated at 37°C for 24 h (Hutchinson et al., 2004).

For *E. coli* O157, a 10 g soil sample was added to 90 ml of modified Tryptone Soya Broth (mTSB; Oxoid, Fanning Ltd., Ireland) with cefixime (50 μg/l) and vancomycin (6 mg/l), stomached for 60 s and incubated at 37°C for 24 h. Immunomagnetic separation (IMS, Dynal® BeadRetriever, Thermo Fisher Scientific, Ireland) was undertaken using Dynabeads™ anti-*E. coli* O157 (Thermo Fisher Scientific, Ireland) and recovered cells were plated on CT-SMAC, incubated at 37°C for 24 h. Suspect colonies were streaked onto both Eosin Methyl Blue agar (EMB, Oxoid, Fannin Ltd, Ireland) and plate count agar (PCA, Oxoid, Fannin Ltd, Ireland), then incubated at 37°C for 24 h. EMB plates were inspected for a green metallic sheen, and corresponding colonies on PCA plates were used to carry out agglutination testing using the Sifin Anti-coli O157 sera test (Cruinn Diagnostics Ltd, Ireland) (International Standards Organisation, 2017).

Low concentrations of *L. monocytogenes* were detected by adding 10 g of the soil sample to 90 ml of half strength Fraser Broth (Oxoid, Fannin Ltd., Ireland) incubated overnight at 30°C, after which 0.1 ml of the resultant culture was added to 10 ml of full-strength Fraser broth (Oxoid, Fannin Ltd., Ireland) and incubated at 37°C for 48 h. Duplicate 100 μl aliquots were plated on *Listeria* selective Oxford Agar (Oxoid, Fannin Ltd., Ireland) with streptomycin sulphate (1,000 μg/ml) and characteristic colonies were considered to be *L. monocytogenes.*

For *E. faecalis*, 10 g of soil sample was combined with 90 ml of BBL enterococcosel broth (Becton Dickinson, Limerick, Ireland) and incubated at 37°C for 24 h before plating on Slanetz and Bartley Agar (Oxoid, Fannin Ltd., Ireland), incubated at 37°C for 24 h and 42°C for a further 24 h. Pink/red colonies were considered to be *E. faecalis*.

Low concentrations of *C. sporogenes* were detected by adding exactly 10 g of soil sample to 90 ml of Reinforced Clostridial Medium (RCM; Oxoid, Fannin Ltd., Ireland) and incubating anaerobically as previously described at 37°C for 48 h. The enrichment culture was then plated on RCA in duplicate, and colonies confirmed using the qPCR method as described above.

Micro-Plot Survival Study

Plot Preparation

An outdoor study site of approximately 0.13 acres (526 m^2) was selected at the Teagasc campus in Ashtown, Dublin (latitude 53.381590, longitude −6.336162) in the east of Ireland. The area has a temperate maritime climate, with an average annual temperature of 10.4°C and mean annual precipitation of 807.6 mm (Met Éireann, 2019). The site was un-grazed and has been covered in a grass sward for many years with regular cutting but no slurry or other fertiliser treatment. The topography was slightly undulating with an average slope of ∼10° along the length and 4° across the width. *Listeria innocua* was selected as the model organism as it is non-pathogenic, absent from the soil samples tested at the site and capable of surviving in soil. Exactly 12 micro-plots were prepared and randomly assigned to three groups (four plots each); (1) control (uninoculated); (2) *L. innocua* applied in digestate; and (3) *L. innocua* applied in bovine slurry.

These micro-plots were prepared as described by Brennan et al. (2012) and Nolan et al. (2020), with the longest dimension in the direction of the slope and laid out using the micro-plot aluminium frame (0.4 x 0.9 m) as shown in **Figure 1**. The frame was hammered into the soil and each plot was a minimum of one frame width apart. The edges of the plot were sealed with clay (to prevent infiltration from outside of the plot area) and a 0.6 m polypropylene plastic run-off collection channel was fitted. After installation, plots were initially left uncovered to allow natural rainfall to wash away any soil disturbed during the process. From the application date, rain out shelters were used to cover plots and exclude natural rainfall (Nolan et al., 2020).

Inoculum Preparation and Application

Three environmental *L. innocua* strains (isolated in fields grazed by cattle) were chosen from the Teagasc culture collection (designated M220, M344 and T1095) and streptomycin sulphate resistant variants of these strains (1,000 μg/ml) (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) were prepared using the method of Blackburn and Davies (1994). Marked strains were stored on cryoprotective beads at −80°C. To prepare an inoculum,

FIGURE 1 | The layout of the micro-plots (left) and a micro-plot with the aluminium frame and collection channel installed (right).

a cryoprotective bead was streaked on TSA and incubated at 37°C for 24 h, after which a single colony was removed using a sterile loop, mixed into 10 ml of tryptone soya broth (TSB; Oxoid, Fannin Ltd., Ireland) and incubated overnight at 37°C. The cultures obtained were centrifuged and washed three times with PBS (Oxoid, Fannin Ltd., Ireland), before resuspension in PBS. The 3 strains were then mixed to create a single *L. innocua* cocktail and serially diluted to give a final concentration of ∼5 $\log_{10}$ cfu/g when inoculated into the slurry or digestate, which was performed as previously described.

Treatment of Soils With Inoculated Slurry or Digestate

To ensure the field trials mimicked reality and that the results were comparable, the amount of slurry and digestate applied to the micro plots was governed by the phosphorous (P) content of these materials and the P index of the soil. All the micro-plots were classified into index 2 soil for grassland crops (soil P range of 3.1 to 5.0 mg/L) so the slurry and digestate were applied at a rate of 40 Kg per hectare (Wall and Plunkett, 2020). Thus 3,838 g of slurry and 1,644 g of digestate were spread on each micro-plot. These were applied in rows using a watering can to mimic application via dribble bar on a slurry spreader (**Figure 2**).

Rainfall was simulated using the Amsterdam drip type simulator (Bowyer-Bower and Burt, 1989) at a target rate of ∼11 mm per plot per h^{-1}, as used in other similar studies (Peyton et al., 2016). The rainfall simulator formed droplets of median diameter of 2.3 mm, spaced 30 mm apart in a 1,000 × 500 × 8 mm perspex plate over a 0.5 m^2 simulator area. To maximise control and to prevent runoff losses due to natural rainfall events, individual micro-plots were covered between treatments by large sheets of plastic. The first rainfall simulation (30 min) took place 24 h after treatment application thus representing a worst-case scenario that is contrary to current regulations, which stipulate that spreading of organic manure should not be carried out within 48 h of forecasted heavy rain [Nitrates Directive (91/676/EEC)]. The second was performed after 48 h (a scenario compliant with current regulations) the third after 14 d and the final rainfall simulation after 30 d. The first 50 ml, the last 50 ml and the runoff in between were collected separately and tested for the *L. innocua* strains using brilliance listeria agar (formerly Oxoid Chromogenic Listeria Agar (OCLA), (Oxoid, Fannin Ltd., Ireland) supplemented with streptomycin sulphate (1,000 μl/g; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and incubated at 37°C for 48 h. Soil samples were taken using a soil plugging tool on the day of the final rainfall simulation (30 d) and also after 58, 86 and 112 d and tested for *L. innocua* (as above).

FIGURE 2 | Inoculated slurry (left) and digestate (right) were applied to the microplots in linear rows.

Data Analysis

The laboratory-based soil survival study was performed in duplicate and repeated on 3 separate occasions. Bacterial counts were converted into $\log_{10}$ cfu/g and the T_{90}-values (the time required to achieve a 90% (1 log) reduction in the population) were determined by linear regression using Graphpad Prism 7 software (San Diego, CA, USA), considering each replicate Y-value as an individual point. Differences between slopes were examined using one-way ANOVA and Tukeys multiple

TABLE 1 | The pH and a_w of the soil samples throughout the experiment.

Time (d)	Sandy soil								Clay soil							
	Slurry				Digestate				Slurry				Digestate			
	4°C		14°C		4°C		14°C		4°C		14°C		4°C		14°C	
	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w
0	7.0	0.99	6.9	0.99	6.5	0.99	6.5	0.99	6.2	0.99	6.7	0.99	6.3	0.99	6.1	0.95
12	6.7	1.00	6.6	1.00	6.9	1.00	5.9	1.00	6.2	1.00	5.6	1.00	6.0	1.00	5.3	1.00
26	6.1	0.99	6.3	0.99	6.1	0.99	5.1	0.99	5.9	1.00	5.6	0.99	5.6	0.99	4.9	0.99
40	5.9	0.99	6.1	0.99	5.9	0.99	5.4	0.99	5.7	0.99	5.6	0.99	5.1	0.99	5.1	0.99
54	6.0	1.00	6.7	0.99	5.8	0.99	5.6	1.00	5.3	0.99	5.7	0.99	5.0	1.00	5.0	1.00
68	6.3	0.99	6.3	0.99	6.1	1.00	5.8	0.99	5.2	0.99	5.6	1.00	5.1	0.99	5.1	1.00
82	6.6	0.99	6.5	1.00	6.2	0.99	5.8	1.00	5.8	1.00	5.9	0.99	5.3	0.99	5.3	1.00
96	6.9	0.99	6.4	1.00	6.4	0.99	5.6	1.00	6.5	0.99	6.4	0.99	5.9	0.99	5.8	1.00
110	6.1	0.99	6.5	0.99	5.7	0.99	5.6	1.00	6.5	0.99	6.2	1.00	5.6	0.99	5.6	1.00
124	6.0	1.00	6.0	0.99	5.3	0.99	5.3	1.00	6.1	0.99	5.9	1.00	5.4	0.99	5.5	0.99

comparison tests (GraphPad Prism 7.02). Statistical significance was set at the 5% level ($P < 0.05$). For the field study, *L. innocua* counts were converted to $\log_{10}$ cfu/ml and differences in the mean counts in each sample type (first 50 ml, last 50 ml and overall) were analysed using one-way ANOVA (Graphpad Prism 7.02).

RESULTS

In the laboratory survival studies the pH of the sandy soil amended with slurry ranged from 5.9 to 7.0. The corresponding figures for sandy soil plus digestate, clay soil plus slurry and clay soil plus digestate were 5.3 to 6.9, 5.2 to 6.7 and 4.9 to 6.3 regardless of bacterial inoculum or storage temperature (**Table 1**). The a_w in all soil-amendment combinations ranged from 0.95 to 1.0, regardless of storage temperature (also **Table 1**).

The decimal reduction curves are shown in **Figures 3**–**7** and the T_{90} values are provided in **Table 2**. Growth was not detected and the decline phase followed first order kinetics for all of the target bacteria regardless of soil type, storage temperature or amendment type. Although there was no shoulders observed, tailing was obtained at 4°C (slurry and digestate) with *S.* Senftenberg (sandy soil), *E. coli* O157:H7 (both soil types) and *E. faecalis* (sandy soil). The T_{90} values obtained for *S.* Senftenberg stored at 4°C were 21.3, 18.5, 17.9 and 16.7 d in sandy-slurry, sandy-digestate, clay-slurry and clay-digestate, respectively. The corresponding T_{90} values for *E. coli* O157:H7 were 25.0, 23.9, 24.4 and 21.7 d, respectively, for *L. monocytogenes* were 11.9, 12.4, 12.8 and 12.1 d, and for *E. faecalis* were 33.3, 30.3, 50.0 and 41.7 d. The reduction in *C. sporogenes* was marginal (1 $\log_{10}$ cfu/g or less over 124 d) and this is reflected in the T_{90} values, which were considerably higher at 166.7, 111.1, 111.1 and 100.0 d. For a given bacterium, the T_{90} values were not significantly ($P > 0.05$) different regardless of the soil type or amendment (slurry or digestate) except for *E. coli* O157 and *E. faecalis,* both in sandy soil where the T_{90} values obtained in slurry-amended soil were significantly higher than the corresponding values in digestate-treated soil.

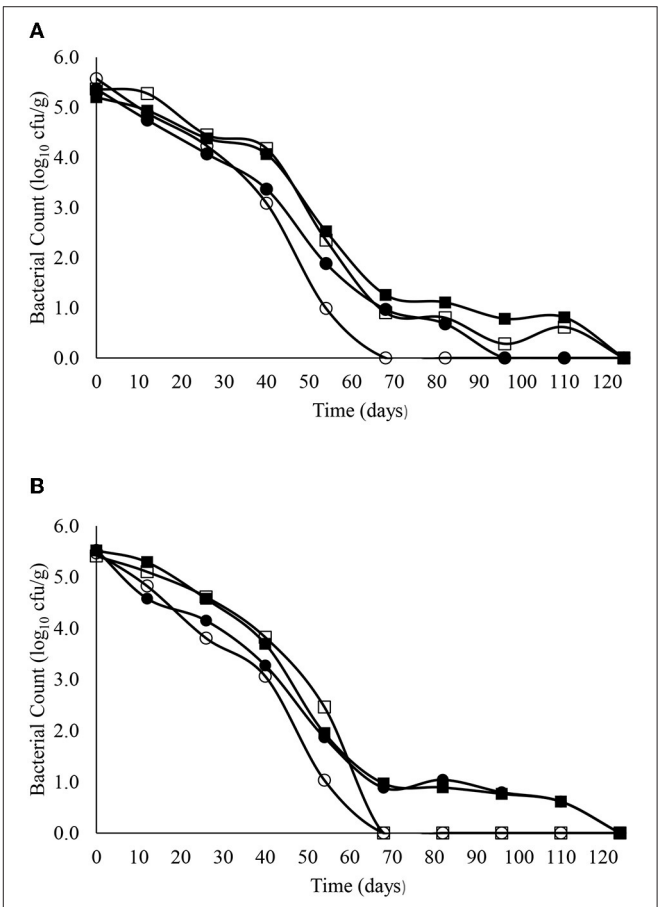

FIGURE 3 | The reduction in *S.* Senftenberg over time in sandy **(A)** and clay **(B)** soils including with slurry at 4°C (■), digestate at 4°C (□), slurry at 14°C (•) and digestate at 14°C (o).

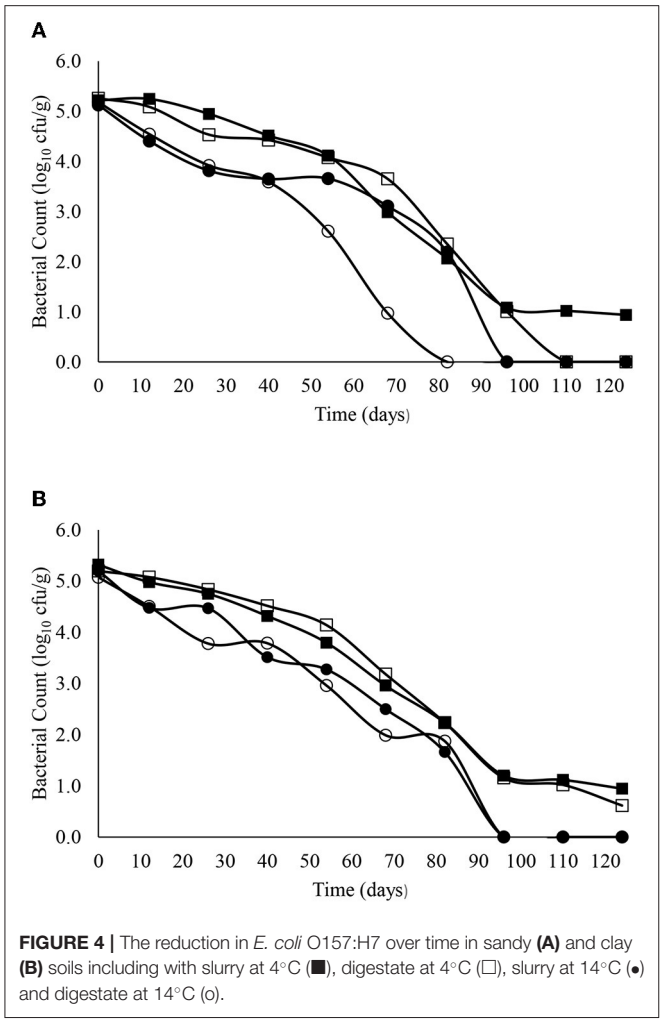

FIGURE 4 | The reduction in *E. coli* O157:H7 over time in sandy **(A)** and clay **(B)** soils including with slurry at 4°C (■), digestate at 4°C (□), slurry at 14°C (●) and digestate at 14°C (○).

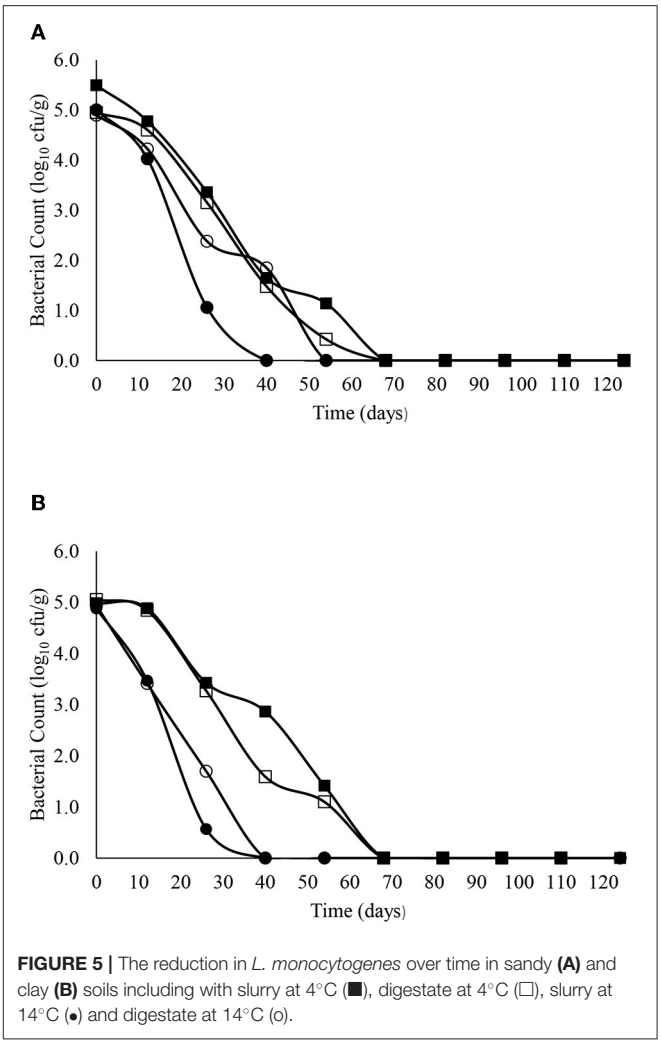

FIGURE 5 | The reduction in *L. monocytogenes* over time in sandy **(A)** and clay **(B)** soils including with slurry at 4°C (■), digestate at 4°C (□), slurry at 14°C (●) and digestate at 14°C (○).

At 14°C the T_{90} values for *S.* Senftenberg, *E. coli* O157:H7, *L. monocytogenes, E. faecalis* and *C. sporogenes* ranged from 12.4 to 20.8 d, 17.6 to 36.6 d, 6.0 to 12.2 d, 18.1 to 36.6 d and 106.5 to 156.0 d, respectively (**Table 3**). For most pathogens soil type or application material had no effect with the exception of *E. coli* O157 and *E. faecalis* in sandy soils where significantly ($P < 0.05$) higher T_{90} values were obtained in slurry compared to digestate.

The uninoculated slurry, digestate and the soil in the field plots all tested negative for *L. innocua* (10 samples each) prior to commencement of the field trial. The initial concentrations of *L. innocua* in the inoculated slurry and digestate was 5 log_{10} cfu/g (data not shown). In slurry and digestate this concentration decreased to 3.1 log_{10} cfu/g and 4.5 log_{10} cfu/g after 2 days, respectively. In both materials the *L. innocua* population remained at this level until at least day 14. Thereafter it was not possible to test the slurry or digestate for *L. innocua* as these materials had become absorbed and mixed into the soil. Soil samples were therefore obtained after day 30 and tested. Indeed, it was advised not to take soil samples before this stage as removal of soil plugs from the plot could influence the way in which the rainfall penetrated the soil and hence the concentrations of *L. innocua* in the run-off. *L. innocua* was not detected in the soil samples taken throughout this experiment at 30, 58, 86 or 112 days (data not shown).

Rainfall was simulated at a rate of ∼11 mm per plot h^{-1} for 30 min, the runoff collected and the bacterial concentration in the first 50 ml, last 50 ml and overall runoff measured. On the first day the *L. innocua* counts from slurry amended soil were 2.4, 2.0 and 2.0 log_{10} cfu/ml, respectively and the corresponding counts when applied in digestate were 1.9, 1.9 and 1.6 log_{10} cfu/ml (**Table 4**). On day 2 the equivalent counts were 2.4, 2.5 and 2.2 log_{10} cfu/ml for slurry and 2.0, 2.0 and 1.7 log_{10} cfu/ml for digestate. On day 14 a similar pattern was observed (2.0, 2.2 and 2.0 log_{10} cfu/ml for slurry vs. 1.7, 1.7 and 1.3 log_{10} cfu/ml for digestate) and again on day 30 (2.4, 2.4 and 1.7 log_{10} cfu/ml for slurry and 1.6, 1.7 and below the level of detection for digestate). Overall, significantly ($P < 0.05$) lower *L. innocua* counts were obtained when applied in digestate on day 1 (first 50 ml and entire sample), day 2 and day 30 (all samples).

DISCUSSION

The T_{90} values obtained for *S.* Senftenberg stored at 4°C ranged from 16.7 to 21.3 d and at 14°C from 12.4 to 20.8 d, regardless

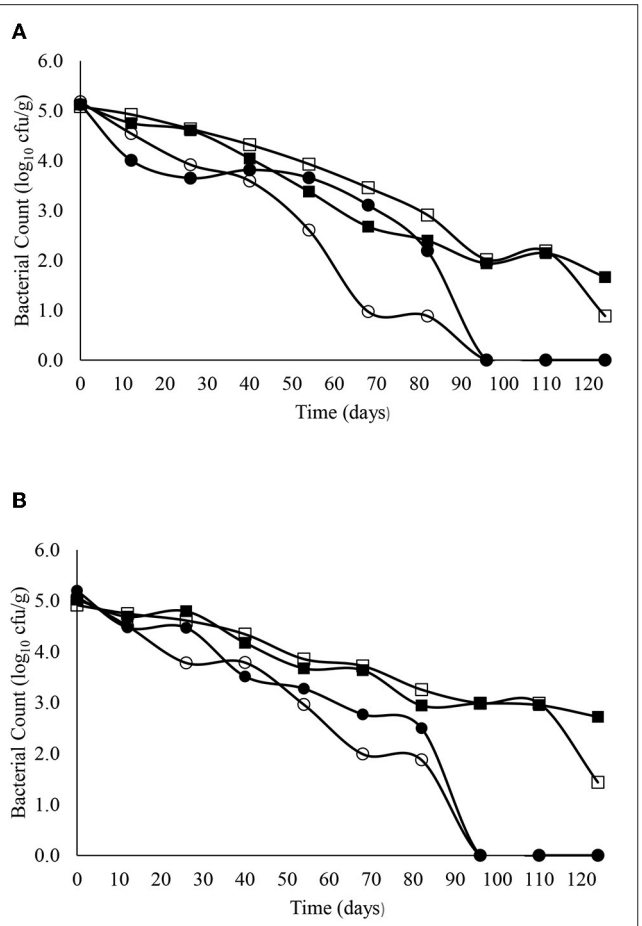

FIGURE 6 | The reduction in *E. faecalis* over time in sandy **(A)** and clay **(B)** soils including with slurry at 4°C (■), digestate at 4°C (□), slurry at 14°C (●) and digestate at 14°C (o).

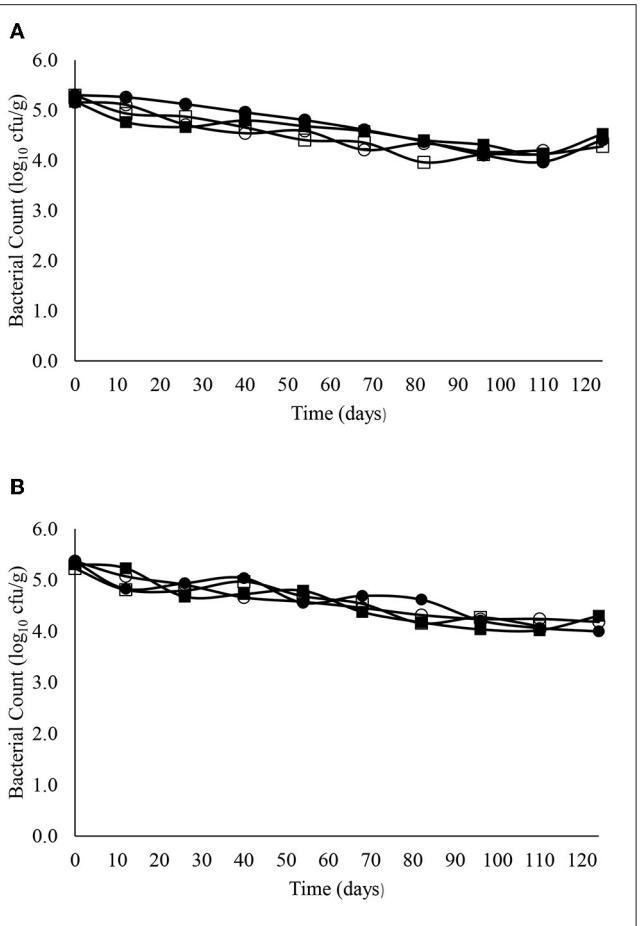

FIGURE 7 | The reduction in *C. sporogenes* over time in sandy **(A)** and clay **(B)** soils including with slurry at 4°C (■), digestate at 4°C (□), slurry at 14°C (●) and digestate at 14°C (o).

of soil type or amendment. Holley et al. (2006) mixed a 6-strain cocktail of *Salmonella* serovars (Agona, Hadar, Heidelberg, Montevideo, Oranienburg, and Typhimurium) into pig manure and added to a Reinfeld loamy sand and a Marquette clay soil before incubation at different temperature sequences. These represented the winter to summer (−18, 4, 10, 25°C), spring to summer (4, 10, 25, 30°C), or summer to winter (25, 10, 4, −18°C) seasonal periods with each temperature step lasting 45 d. In all cases the T_{90} values were ≤ 30 d which was consistent with our findings. Nicholson et al. (2005) inoculated bovine slurry with *Salmonella* Typhimurium before spreading on agricultural land in summer (temperatures were ≤20°C). These bacteria generally survived for up to 1 month when applied to both sandy and clay soils. Our findings therefore support the hypothesis that *Salmonella* spp. die-off within approximately 4 weeks when applied to land, regardless of soil type, amendment (manure, slurry or digestate) or temperature.

The T_{90} values for *E. coli* O157:H7 ranged from 21.7 to 25.0 d at 4°C and from 17.6 to 36.6 d at 14°C. In contrast, the Nicholson et al. (2005) study mentioned above, reported an approximate 3.5 log_{10} cfu/g reduction in *E. coli* O157:H7 in 4 days when applied in bovine slurry to sandy soils but a residual population of ∼0.3 log_{10}cfu/g persisted until day 25. After a short initial growth (increase from 3.5 to 4.0 log_{10} cfu/g) in slurry spread on clay soils, the *E. coli* O157:H7 decrease followed first order kinetics and was not detected after 35 days. Previous research by our group investigated the survival of a non-toxigenic strain of *E. coli* O157:H7 in bovine faeces spread on grassland. A 4.0–5.0 log_{10} cfu/g reduction was observed within 50 d but the organism was still detectable in the surrounding soil for up to 99 d (Bolton et al., 1999). More recently our group also examined the survival of 6 bovine non-O157 Shiga toxin-producing *E. coli* (STEC) in sandy and clay soils at 10°C for up to 201 days and obtained T_{90} values that ranged from 50·3 to 75·6 days in sandy soils and from 31.6 to 48.3 days in clay soils (Bolton et al., 2011). Thus, there is no consistency in the T_{90} values reported for *E. coli* O157:H7 or STEC survival in soil possibly due to differences in soil composition, temperature, pH, moisture, exposure to UV light from the sun, protozoan predation and experimental design (Hutchinson et al., 2004; Franz et al., 2005; Jacobsen and Bech, 2012).

The corresponding T_{90} values for *L. monocytogenes*, *E. faecalis* and *C. sporogenes* ranged from 11.9 to 12.8 d, 30.3 to 50.0 d and 100 to 166.7 d (regardless of soil type or amendment), respectively at 4°C and from 6.0 to 12.2 d, 18.1 to 36.6 d and

TABLE 2 | T_{90} values for the different bacteria applied in slurry and digestate to sandy loam and clay loam soils and stored at 4°C in the laboratory trials.

Bacteria	Conditions			Decimal reduction				
	T (°C)	Soil type	Slurry or digestate	R^2	Slope	SE	*n*	T_{90} (days)
S. Senftenberg	4	Sandy	Slurry	0.91	−0.047	0.0005	54	$21.3^{A/A}$
	4	Sandy	Digestate	0.91	−0.054	0.0064	54	$18.5^{A/A}$
	4	Clay	Slurry	0.94	−0.056	0.0052	54	$17.9^{A/A}$
	4	Clay	Digestate	0.91	−0.060	0.0076	54	$16.7^{A/A}$
E. coli O157	4	Sandy	Slurry	0.97	−0.040	0.0025	60	$25.0^{A/A}$
	4	sandy	digestate	0.95	−0.042	0.0033	60	$23.9^{A/A}$
	4	Clay	Slurry	0.93	−0.041	0.0039	60	$24.4^{A/A}$
	4	Clay	Digestate	0.91	−0.046	0.0052	60	$21.7^{A/A}$
L. monocytogenes	4	Sandy	Slurry	0.98	−0.084	0.0054	24	$11.9^{A/A}$
	4	Sandy	Digestate	0.97	−0.081	0.0071	24	$12.4^{A/A}$
	4	Clay	Slurry	0.97	−0.078	0.0071	36	$12.8^{A/A}$
	4	Clay	Digestate	0.94	−0.083	0.0100	36	$12.1^{A/A}$
E. faecalis	4	Sandy	Slurry	0.96	−0.030	0.0022	60	$33.3^{A/A}$
	4	Sandy	Digestate	0.96	−0.033	0.0025	60	$30.3^{A/A}$
	4	Clay	Slurry	0.91	−0.020	0.0019	60	$50.0^{A/A}$
	4	Clay	Digestate	0.94	−0.024	0.0027	60	$41.7^{A/A}$
C. sporogenes	4	Sandy	Slurry	0.70	−0.006	0.0013	60	$166.7^{A/A}$
	4	Sandy	Digestate	0.78	−0.009	0.0017	60	$111.1^{A/A}$
	4	Clay	Slurry	0.72	−0.009	0.0020	60	$111.1^{A/A}$
	4	Clay	Digestate	0.78	−0.010	0.0018	60	$100.0^{A/A}$

Statistical analysis: The first capital letter refers to the comparison of sandy vs. clay with the application material (slurry or digestate) being constant. The second capital letter refers to the comparison of slurry vs. digestate with the soil type (sandy or clay) kept constant. Different letters indicate statistical difference at the 5% level ($P < 0.05$).

TABLE 3 | T_{90} values for the different bacteria applied in slurry and digestate to sandy loam and clay loam soils and stored at 14°C in the laboratory trials.

Bacteria	Conditions			Decimal reduction				
	T (°C)	Soil type	Slurry or digestate	R^2	Slope	SE	*n*	T_{90} (days)
S. Senftenberg	14	Sandy	Slurry	0.91	−0.061	0.0058	54	$16.4^{A/A}$
	14	Sandy	Digestate	0.91	−0.081	0.0118	54	$12.4^{A/A}$
	14	Clay	Slurry	0.94	−0.048	0.0065	54	$20.8^{A/A}$
	14	Clay	Digestate	0.91	−0.079	0.0096	54	$12.7^{A/A}$
E. coli O157	14	Sandy	Slurry	0.97	−0.027	0.0054	60	$36.6^{A/B}$
	14	Sandy	Digestate	0.95	−0.057	0.0072	60	$17.6^{A/A}$
	14	Clay	Slurry	0.93	−0.039	0.0065	60	$25.8^{A/A}$
	14	Clay	Digestate	0.91	−0.040	0.0034	60	$25^{A/A}$
L. monocytogenes	14	Sandy	Slurry	0.98	−0.153	0.0375	36	$6.5^{A/A}$
	14	Sandy	Digestate	0.97	−0.082	0.0127	36	$12.2^{A/A}$
	14	Clay	Slurry	0.97	−0.167	0.0257	36	$6.0^{A/A}$
	14	Clay	Digestate	0.94	−0.125	0.0013	36	$8.0^{A/A}$
E. faecalis	14	Sandy	Slurry	0.96	−0.027	0.0054	60	$36.6^{A/B}$
	14	Sandy	Digestate	0.96	−0.055	0.0051	60	$18.1^{A/A}$
	14	Clay	Slurry	0.91	−0.030	0.0064	60	$33.2^{A/A}$
	14	Clay	Digestate	0.94	−0.040	0.0034	60	$25^{A/A}$
C. sporogenes	14	Sandy	Slurry	0.70	−0.008	0.0027	60	$121.7^{A/A}$
	14	Sandy	Digestate	0.78	−0.006	0.0025	60	$156.0^{A/A}$
	14	Clay	Slurry	0.72	−0.009	0.0016	60	$106.5^{A/A}$
	14	Clay	Digestate	0.78	−0.007	0.0019	60	$137.9^{A/A}$

Statistical analysis: The first capital letter refers to the comparison of sandy vs. clay with the application material (slurry or digestate) being constant. The second capital letter refers to the comparison of slurry vs. digestate with the soil type (sandy or clay) kept constant. Different letters indicate statistical difference at the 5% level ($P < 0.05$).

TABLE 4 | The concentrations of *L. innocua* in leachate during rainfall simulation from inoculated slurry and digestate.

Time (days)	Sample type	Slurry			Digestate			*P*-value	Significant ($P < 0.05$)
		mean	SEM	*n*	mean	SEM	*n*		
					Runoff				
1	First 50 ml	2.4	0.108	4	1.9	0.061	12	0.0243	Yes
	Last 50 ml[a]	2.0	0.308	4	1.9	0.071	12	0.8065	No
	Entire sample	2.0	0.066	4	1.6	0.052	12	0.0227	Yes
2	First 50 ml	2.4	0.172	4	2.0	0.063	12	0.1033	Yes
	Last 50 ml	2.5	0.141	4	2.0	0.086	12	0.0397	Yes
	Entire sample	2.2	0.099	4	1.7	0.083	12	0.0178	Yes
14	First 50 ml	2.0	0.305	4	1.7	0.194	12	0.3890	No
	Last 50 ml	2.2	0.393	4	1.7	0.196	12	0.2834	No
	Entire sample	2.0	0.301	4	1.3	0.382	12	0.1991	No
30	First 50 ml	2.4	0.076	4	1.6	0.0256	12	0.0093	Yes
	Last 50 ml	2.4	0.051	4	1.7	0.014	12	0.0044	Yes
	Entire sample	1.7	0.262	4	ND	NA	12	NA	Yes
58	First 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Last 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Entire sample	ND	NA	4	ND	NA	12	NA	NA
86	First 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Last 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Entire sample	ND	NA	4	ND	NA	12	NA	NA
112	First 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Last 50 ml	ND	NA	4	ND	NA	12	NA	NA
	Entire sample	ND	NA	4	ND	NA	12	NA	NA

[a]*After 30 min rain.*

106.5 to 156.0 d, respectively, at 14°C. Nicholson et al. (2005) reported a decrease from 2.5 $\log_{10}$ cfu/g to "not detected" in sandy soil after 8 days but, as with *E. coli* O157:H7, the decrease on clay soil was almost linear, with the same decrease requiring 32 days. Although *E. faecalis* and *Clostridium* spp. are common in bovine faeces and digestate (Russell et al., 2020), studies on their survival in amended soils are limited. Byappanahalli and Fujioka (2004) reported that *Enterococci* are well-adapted to survive in soil, while Sinton et al. (2007) reported a decimal reduction of *Enterococci* after 56 days in bovine faeces on pasture. Our data suggests *Clostridium* spp. persist for extended periods in soil. This was not unexpected as soil is the natural habitat for *Clostridium* spp. such *as Clostridium botulinum* and inoculation studies in soil have demonstrated persistence for several months (Gessler and Böhnel, 2006). Moreover, Girardin et al. (2005) reported that *C. sporogenes* inoculated into soil in field trials declined by <0.7 log after 16 months.

With the exception *E. coli* O157:H7 and *E. faecalis,* in sandy soil (where the T_{90} values obtained were significantly higher for slurry as compared to digestate-amended soil), the T_{90} values were not significantly ($P > 0.05$) different regardless of the soil type or application material (slurry or digestate). Thus, our findings are more or less consistent with those of Saunders et al. (2012), who reported no significant difference in the die-off rate of *E. coli* and faecal coliforms in slurry when compared to digestate obtained from the anaerobic digestion of dairy waste). Given the temperature, pH and a_w were similar, the significantly reduced survival of *E. coli* O157:H7 and *E. faecalis* in digestate amended soil may be the result of lower organic matter and/or nutrient levels (García-Orenes et al., 2010; Nolan et al., 2020). Moreover, the concentration of volatile acids and microbial diversity may also play a role (Klein et al., 2011; Orzi et al., 2015). However, further research would be required to confirm this hypothesis and to determine whether the effects are direct or indirectly via changes in the indigenous soil microbial community (Vinten et al., 2002).

The storage temperatures used in our study (4°C and 14°C) did not appear to exert a consistent effect on bacterial survival. Of the twenty inoculated soil combinations, the T_{90} values were higher at 4°C on 12 occasions, similar twice and lower for the remainder (6). Phan-Thien et al. (2020) investigated the effects of soil type and temperature on the survival of a cocktail of 5 *Salmonella* enterica serotypes (Enteritidis, Infantis, Montevideo, Typhimurium and Zanzibar) in poultry manure amended sandy and clay soils at 5, 21 and 37°C for 6 weeks. Higher reductions (3 to 4 $\log_{10}$cfu/g) were obtained at the higher temperature while a 1 to 2 log reduction was observed at 5°C. Another similar study reported that *Salmonella* Typhimurium survived better in topsoil at 5°C as compared to 15 or 25°C (García-Orenes et al., 2010). Moynihan et al. (2013) also observed the maintenance of a cocktail of 2 *E. coli* O157:H7 strains at 4°C in Bearsted soil (a typical brown earth, pH 6.07) and Evesham

soil (a typical calcareous pelosol, described, pH 6.6). However, field studies suggest that *E. coli* levels decline in soil when the temperature decreases below 5°C, although the *L. innocua* persists, independent of temperature (Reed-Jones et al., 2016).

A key finding of our field trial was the retention of *L. innocua* in digestate resulting in significantly ($P < 0.05$) lower *L. innocua* counts in the run-off on day 1 (first 50 ml and entire sample), day 2 and day 30 (all samples). During rainfall, the water either infiltrates into the soil and may be bound by the matrix within gel-like structures or runs off with the latter being more important in terms of the risk of contamination of adjacent water and/or crops (Gentry et al., 2017). This is particularly important when heavy rainfall causes flooding that carries pathogens to adjacent fields and water bodies (Steele and Odumeru, 2004). Water flow in soil systems is complex and can be influenced by soil (including organic matter such as slurry or digestate) texture, structure, hydraulic properties, slope and cover (Jacobsen and Bech, 2012). In addition to the properties of the soil, cellular characteristics such as size, electric charge and hydrophobicity can influence bacterial concentrations found in run-off (Jacobsen and Bech, 2012).

In general, adding organic matter to soil improves the soil structure and fertility (Frøseth et al., 2014). However, the effect is dependent on the type of organic matter with some organic waste materials increasing porosity and water permeability while other have the opposite effect (De Gryze et al., 2006; Benito et al., 2016). Thus, although several studies have demonstrated the dissemination of faecal bacteria from contaminated material on the soil surface to both surface and ground water (Vinten et al., 2002; Gentry et al., 2017), the results may not be comparable to those obtained with *L. innocua* in this study. Pastorelli et al. (2021) observed an improvement in soil aggregate stability when digestate was added but the proportion of transmission pores and fissures decreased thereby decreasing soil permeability. Digestates also contain higher concentrations of hydrophobic components than cattle slurry (Šimon et al., 2016). Thus, we hypothesise that rainwater falling on freshly applied digestate (as compared to slurry) will have lower penetration and reduced ability to remove bacteria in the resultant run-off. Furthermore, over time the permeability of the digestate amended soil may be less than slurry-treated pastures, reducing leeching of any surviving bacteria into ground water, but thorough investigation is required.

CONCLUSION

It was concluded that with exceptions [*E. coli* O157:H7 and *E. faecalis* in sandy soil (slurry vs. digestate)], the type of organic fertiliser (slurry vs. unpasteurised digestate) does not affect survival rates of the bacteria tested in soil. However, a higher proportion of bacteria may be retained in the soil-digestate matrix during periods of rainfall, thereby reducing the risk of ground and surface water contamination.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

DB was responsible for conceiving and the design of the study and obtained the funding. LR acquired and analysed and interpreted the data. DB drafted the manuscript while LR, PW, AZ, SG, BM, TW, SN, VO'F, FA, KR, and OF reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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