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Survival of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cow manure and cow manure slurry

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Abstract

An exponential linear destruction was observed for *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cattle manure and manure slurry stored at 4, 20 or 37°C. The resulting decimal reduction times ranged from 6 days to 3 weeks in manure and from 2 days to 5 weeks in manure slurry. The main effects of time as well as temperature were pronounced with the most rapid destruction at 37°C. The ammonia concentration in manure increased slightly during storage but did not exceed 0.1%. pH values in the deeper layers of manure remained constant except at 37°C when the pH increased by 1 unit in 60 days. In the surface layers of manure, pH increased by 1.5–2 units, the oxidation-reduction potential of the manure declined rapidly to values below −200 mV. These changes do not seem to be reflected in changing rates of bacterial destruction. The observed order of destruction makes it possible to predict storage conditions (temperature and time) that will lead to a predetermined level of reduction of the two pathogens.

Keywords: Manure; Survival; *Salmonella*; *Escherichia coli* O157:H7

1. Introduction

Human infections with *Salmonella* are common throughout the world [1]. Cattle may serve as a source of both *Salmonella* and *Escherichia coli* O157:H7. Cattle may shed the bacteria through milk and feces without showing any clinical signs [2–4].

Manure, which consists of animal excreta (feces and urine) mixed with bedding and sometimes diluted with water, may also contain secretions from nose, throat, vagina, blood, mammary gland, skin and placenta [2]. Feces from colonized cows may contain from 10^2 to 10^7 colony forming units (CFU) *Salmonella* cells g^{-1} of feces. Cattle, particularly calves and heifers colonized with *E. coli* O157:H7 may shed the organism at levels ranging from 10^2 to 10^5 CFU g^{-1}. Among dairy herds in the northwestern of USA, 75% were fecal positive for *E. coli* O157:H7 [5]. A survey of feedlots in 13 states of the USA demonstrated the presence of *E. coli* O157:H7 in 63% [6].

Contaminated manure and manure slurry may pose a risk when used as a fertilizer for crops. Several conditions may influence the survival of patho-

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gens in manure or slurry. They include temperature, pH, solids content, microbial content, oxidation-reduction potential (ORP) and time. However, few reports attempting to quantify the impact of these factors on survival of *Salmonella* or *E. coli* O157:H7 in manure were found. The objective of this study was to measure the impact of a few selected factors on the growth/survival of *E. coli* O157:H7 and *Salmonella typhimurium* in cow manure and manure slurry.

2. Materials and methods

2.1. Bacterial strains

The strains of bacteria used were *E. coli* O157:H7 (ATCC 43890, batch 3-300-XH, Rockville, MD, USA) and *S. typhimurium* isolated from a California poultry house. These organisms were genetically modified by inserting plasmids (Clontech, San Francisco, CA, USA) encoding synthesis of protein that fluoresces green (GFP) or blue (BFP), respectively, when illuminated with 365-nm wavelength ultraviolet (UV) light, and for resistance to ampicillin. The GFP plasmid was placed in *E. coli* O157:H7 and the BFP plasmid in *S. typhimurium* using the method described by Hanahan [7]. The plasmid makes it possible to count colonies of the test bacteria in the presence of a large indigenous microflora on media containing ampicillin (100 mg l^{-1}) and novobiocin (20 mg l^{-1}) and to distinguish between *E. coli* O157:H7 and *S. typhimurium* colonies. The bacteria were propagated in brain heart infusion (BHI) broth (Difco®) containing ampicillin and novobiocin at 37°C for 24 h. The cultures were centrifuged and the cells suspended in sterile saline solution before mixing into manure or manure slurry. The inocula were prepared to result in 10^6–10^8 CFU g^{-1} or ml^{-1}.

Tests confirmed that these molecular constructs behave similarly as their parent strains in manure (data not shown).

2.2. Media preparation

BHI agar was prepared by adding 1.5% agar (Difco®) into BHI broth (Difco®). Novobiocin (sodium salt 90%, Sigma Chemicals, St. Louis. MO, USA) and ampicillin (sterile ampicillin sodium, USP, Marsan Pharmaceuticals, Cherry Hill, NJ, USA) were added to the cooled medium (50°C) at concentrations of 20 and 100 mg l^{-1}, respectively.

2.3. Cow manure slurry

Fresh cow manure was collected in plastic bags from the dairy barn, University of California (Davis, CA, USA). It was collected immediately after being deposited and transferred to the laboratory within 10 min. Manure slurry was prepared as described below. Old manure from cows was collected in the field where it had remained for weeks or months and had become compacted and dried.

Manure slurry was prepared by mixing in a plastic bag manure (fresh or old) and deionized water at a ratio of 1:2, similar to slurry sprayed on fields (T. Suslow, personal communication). Insoluble material was allowed to settle and supernatant was collected. Slurries from fresh and old manure were prepared separately. After *E. coli* O157:H7 GFP and *S. typhimurium* BFP were inoculated into the supernatant, 10 ml was pipetted into Whirl-Pak bags (Whirl-Pak®, Nasco, USA) and stored at 4, 20 or 37°C. Duplicate bags were removed at time intervals for bacterial counts and analysis.

2.4. Cow manure

Approximately 6 kg of fresh manure was collected from the dairy barn, University of California (Davis, CA, USA). The manure was mixed and inoculated during mixing with *E. coli* O157:H7 GFP and *S. typhimurium* BFP, using a ratio of 1 ml per 100 g of manure. The manure was then packed in Whirl-Pak bags, each containing 120 g of manure to provide an 8-cm deep layer. The open bags were placed in incubators at temperatures of 4°C (75% RH), 20°C (50% RH) and 37°C (30% RH). Duplicate samples were taken for analysis from the top, middle and bottom locations in each bag. The top sample was 0.5-cm deep starting from the surface, while the middle and the bottom samples were from the center and the very bottom of the bag. A hole was made at each level in the plastic bag with a hot metal cork borer and the sample was collected with a sterile spatula.

2.5. Bacterial counts

A 0.5-g sample of slurry or manure was mixed with 2 ml of sterile normal saline on a vortex mixer. 10-Fold dilutions were prepared in sterile, normal saline and surface-plated on BHI agar containing ampicillin and novobiocin. Colonies were counted after incubation 24 h at 37°C and recorded by their color under the UV lights: green for *E. coli* O157:H7 and blue for *S. typhimurium*. If colonies were absent, a most probable number technique was used to estimate low numbers of bacteria by inoculating 10 ml or g of slurry or manure into 40 ml buffered peptone solution. Four or five additional 10-fold dilutions were made with two replicates per dilution level. The buffered peptone cultures were inoculated at 37°C for 20–24 h and then streaked on the BHI+ampicillin+novobiocin agar plates. The agar plates were checked after incubation at 37°C for 24 h for fluorescent colonies using UV light. The number of bacteria was estimated using Fisher and Yates' procedure [8]. The counts were expressed as log CFU ml^{-1} for slurry and as log CFU g^{-1} dry matter for manure.

2.6. Determination of moisture, ammonia, pH and ORP

Moisture was determined by drying at 105°C for 1 h. Ammonia was determined with an ammonia electrode (Orion®, 95-12 ammonia electrode, Orion Research, Beverly, MA, USA) and results were reported as the percentage of ammonia in a sample. The pH was measured using a pH electrode (Orion® pH electrode 9165 BN, Orion Research, Boston, MA, USA). The ORP was measured by an ORP electrode (Cole-Parmer, Vernon Hills, IL, USA).

2.7. Statistical procedure

Analysis of variance (ANOVA) was carried out with two grouping factors (day and time) and one repeated factor (location) in the manure experiment. The software used was BMDP 2V (ANOVA and repeated measures). In the slurry experiment, a two-way ANOVA was applied and the software used was BMDP 7D (one- and two-way ANOVA with data screening). Linear regression was carried out using the Microsoft Excel® statistical function.

3. Results

3.1. Cow manure

3.1.1. Bacterial counts

The population of the *E. coli* O157:H7 GFP in the top layers increased slightly for the first 3 days at 37°C before decreasing by six log units CFU on day 38. *S. typhimurium* BFP also showed an increase until day 3, before it gradually decreased by six log units CFU on day 48. *E. coli* O157:H7 GFP and *S. typhimurium* BFP exhibited a greater survival at 20°C compared to 4 or 37°C. Comparison of survival between *E. coli* O157:H7 GFP and *S. typhimurium*

Table 1
Rate of microbial destruction in cow manure, regression of the log number survivors on storage time

Organism	Location	Temperature (°C)	Regression equation	R^2	DRT (days)
E. coli O157:H7	Top	4	Y = −0.111X+9.119	0.71	9.04
S. typhimurium	Top	4	$Y = -0.079X+7.468$	0.89	12.70
E. coli O157:H7	Top	20	$Y = -0.046X+9.464$	0.68	21.60
S. typhimurium	Top	20	$Y = -0.040X+7.926$	0.39	24.69
E. coli O157:H7	Top	37	$Y = -0.112X+8.773$	0.56	8.91
S. typhimurium	Top	37	$Y = -0.120X+6.751$	0.56	8.36
E. coli O157:H7	Average (middle+bottom)	4	$Y = -0.054X+8.737$	0.53	18.59
S. typhimurium	Average (middle+bottom)	4	$Y = -0.049X+7.400$	0.81	20.33
E. coli O157:H7	Average (middle+bottom)	20	$Y = -0.074X+9.754$	0.93	13.51
S. typhimurium	Average (middle+bottom)	20	$Y = -0.107X+9.076$	0.86	9.36
E. coli O157:H7	Average (middle+bottom)	37	$Y = -0.279X+9.285$	0.78	3.58
S. typhimurium	Average (middle+bottom)	37	$Y = -0.578X+8.172$	0.99	1.73

Table 2
Values of ammonia, pH, moisture and ORP in manure during storage

Temperature (°C)	Location	Ammonia %		pH		Moisture %		ORP (mV)
		Beginning	End	Beginning	End	Beginning	End	End
4°C	Top layer	0.02	0.04	7.42	8.84	87.6	79.6	ND
	Middle layer	0.02	0.02	7.42	7.26	87.6	85.4	<-200
	Bottom layer	0.02	0.02	7.42	7.10	87.6	85.8	<-200
20°C	Top layer	0.02	0.06	7.42	8.97	87.6	59.3	ND
	Middle layer	0.02	0.09	7.42	7.39	87.6	86.6	<-200
	Bottom layer	0.02	0.10	7.42	7.17	87.6	86.2	<-200
37°C	Top layer	0.02	0.07	7.42	9.47	87.6	11.9	ND
	Middle layer	0.02	0.06	7.42	8.73	87.6	87.6	<-200
	Bottom layer	0.02	0.04	7.42	8.54	87.6	88.6	<-200

BFP in the top layer of manure suggests that *E. coli* O157:H7 GFP had a greater survivability at all the temperatures. In the middle-bottom layers, both bacteria showed an increase in numbers at 20°C during the first 3 days, followed by a decline. Regression analysis indicates a first order inactivation rate, except for the first 3 days, resulting in a linear relationship between time and logs survivors at all temperatures. This permits estimation of the times required for 90% reduction or decimal reduction times (DRT). The regression equations, the associated R^2 values and the derived DRT values are shown in Table 1.

There was no statistically significant difference in the survival of *E. coli* O157:H7 GFP and *S. typhimurium* BFP between the middle and bottom samples. However, there were significant differences between the top and middle and the top and bottom locations. This also applied to the pH and moisture content. Therefore, the data for the middle and the bottom samples were pooled and expressed as an average. Time and temperature had significant effects and a significant interaction with respect to survival of both pathogens (Tables 1–3).

3.1.2. Ammonia, pH, moisture and ORP

The results are summarized in Table 2.

For ammonia, there was a statistically significant ($P<0.0001$) three-way interaction between time, temperature and either pH or moisture. In terms of location, the same pattern as for bacteria was found with a statistically significant difference between top and middle and top and bottom, but not between middle and bottom. No interaction was found between the time and temperature ($P=0.0755$). But significant main effects were found for time, temperatures and location ($P=0.0158$, <0.0001 and 0.0338, respectively). However, the observed differences seem to be of little biological importance since the initial ammonia level was low (0.02%) and the S.E.M.s were ranging from 0.01 to 0.03%.

The initial pH of the manure was 7.42. In the top samples, the pH increased gradually to above 8.5 with the highest value of 9.47 at 37°C. In the middle-bottom portion of the samples, the pH increased above 8.5 at 37°C, while it declined slightly at 4 and 20°C.

The moisture level at the start of the experiment was 88% and decreased at 37°C to 12% in the top

Table 3
Rates of microbial destruction in slurry of fresh cow manure, regression of the log survivors on storage time

Organism	Material	Temperature (°C)	Regression equation	R^2	DRT (days)
E. coli O157:H7	Slurry	4	$Y=-0.046X+6.088$	0.91	21.51
S. typhimurium	Slurry	4	$Y=-0.061X+6.090$	0.93	16.42
E. coli O157:H7	Slurry	20	$Y=-0.068X+8.788$	0.97	14.75
S. typhimurium	Slurry	20	$Y=-0.079X+8.142$	0.78	12.69
E. coli O157:H7	Slurry	37	$Y=-0.315X+7.642$	0.91	3.18
S. typhimurium	Slurry	37	$Y=-0.422X+8.039$	0.99	2.37

Table 4
Rates of microbial destruction in slurry of old cow manure, regression of the log survivors on storage time

Organism	Material	Temperature (°C)	Regression equation	R^2	DRT (days)
E. coli O157:H7	Slurry	4	$Y=-0.026X+6.402$	0.81	38.76
S. typhimurium	Slurry	4	$Y=-0.015X+6.129$	0.64	65.79
E. coli O157:H7	Slurry	20	$Y=-0.130X+6.470$	0.91	7.67
S. typhimurium	Slurry	20	$Y=-0.358X+7.327$	0.95	2.80
E. coli O157:H7	Slurry	37	$Y=-0.443X+5.028$	0.92	2.25
S. typhimurium	Slurry	37	$Y=-0.396X+4.550$	0.93	2.52

samples. At the other temperatures, it ranged between 70 and 90%. In the middle-bottom layers, the moisture was consistently between 84 and 90%.

The ORP ranged between −70 and −230 mV, indicating anaerobic conditions in the middle-bottom layers.

3.2. Slurry

3.2.1. Fresh manure slurry

The bacterial counts are shown in Table 3 and the analytical data in Table 5. Like for manure, a linear relationship between time and log surviving organisms was demonstrated, the initial growth or 'shoulder' on the survivor curve was observed at 20 and 37°C. The rate of destruction was highest at 37°C, followed by 20 and 4°C.

E. coli O157:H7 GFP became non-detectable by day 27 at 37°C. At 20°C, a two log reduction was observed by day 35. At 4°C, the numbers had decreased by three log units on day 60.

For *S. typhimurium* BFP, the slurry became negative at 37°C by day 19. At 4 and 20°C, the population decreased by 1.5 and four log units on day 60. Statistical analysis showed a three-way interaction between microorganism, time and temperature ($P=0.0001$). Because the initial level and the mean of ammonia remained constant at 0.02%, variance was not computable. As to pH, there was an interaction between the temperature and time ($P=0.0001$). At 20°C and 37°C, the pH increased and at 4°C, the readings were fairly constant.

3.2.2. Old manure slurry

In the slurry of old manure, the survival trends were the same as for fresh manure slurry. The DRT values at 4°C were longer in old manure slurry compared to fresh manure slurry, while the opposite was the case for 20 and 37°C (Table 4).

Statistical results for ammonia were the same as those found in fresh cow manure slurry. The initial pH in old manure slurry was 9.03. On day 1, the pH dropped before it started to increase at all temperatures. The increase was noticeable at 20 and 37°C, where the pH reached a value of more than eight. At 4°C, the pH remained fairly constant. Statistical

Table 5
Values of ammonia and pH in slurries from fresh and old cow manure

Temperature (°C)	Material	Ammonia %		pH	
		Beginning[a]	End	Beginning	End
4°C	Fresh manure slurry	0.02	0.05[b]	7.45	7.95[b]
	Old manure slurry	0.03	0.03[b]	9.03	8.19[b]
20°C	Fresh manure slurry	0.02	0.03[b]	7.45	8.58[b]
	Old manure slurry	0.03	0.02[c]	9.03	8.35[c]
37°C	Fresh manure slurry	0.02	0.03[c]	7.45	8.51[c]
	Old manure slurry	0.03	0.01[c]	9.03	8.71[c]

[a]Day 0.
[b]Day 60.
[c]Day 19.

analysis demonstrated an interaction between temperatures and time with respect to pH changes.

4. Discussion

With the exception of an occasional initial multiplication of the test organisms, the rate of inactivation corresponded to a first order reaction with fairly well-defined decimal reduction times. The data presented by Wang et al. [9] also indicated a first order inactivation at 5°C but at 22 and 37°C, the inactivation rate declined over time. This may be due to a decline in water activity to 0.39–0.46. At 5°C, the water activity remained at 0.99.

Zhai et al. [10] observed a two stage first order mortality for fecal coliforms which may be due to a heterogeneous distribution of resistance among the cells of the target organisms or due to changes in environmental conditions.

In the present study, except for 4°C, the inactivation rate was lower for the top layer of manure compared to the middle-bottom layers and the inactivation in the top layer was much slower than reported earlier for thin layers of manure exposed to different levels of relative humidity [11]. The reason is probably that the dehydration rate was much faster in the thin layers of manure. It has been shown that a fast dehydration process results in increased microbial inactivation [12]. On the other hand, long term survival is improved by a low a_w and maximum survival of dried cells is expected when the dehydration rate is slow and the ultimate water activity low. It remains uncertain what the inactivation mechanism is in deeper layers of manure where the water activity remains high. It could be a lack of nutrients caused by a dense population of indigenous microorganisms in the manure or some of these microorganisms may produce compounds detrimental to *S. typhimurium* and *E. coli* O157:H7.

A number of interactions that involve temperature and time were found for the pH and ammonia concentration. These interactions are probably due to the activities of different fractions of the indigenous microflora but their magnitudes seem to be too small to be biologically important for the survival of the test organisms.

DRT values may be used in risk assessments to predict how long manure should be held before application to a field used to grow and produce safe. If for example it is decided that a $\geq 10^5$-fold reduction of *E. coli* O157:H7 and *S. typhimurium* is required in order to make manure safe, then, the manure should be stored for 105 days at 4°C or 45 days at 37°C. Higher temperatures generated in manure piles would accelerate destruction but were not evaluated in the present study.

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