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Assessing the impacts of temperature and storage on *Escherichia coli*, *Salmonella*, and *L. monocytogenes* decay in dairy manure

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Abstract Elevated levels of animal waste-borne pathogen in ambient water is a serious human health issue. Mitigating influx of pathogens from animal waste such as dairy manure to soil and water requires improving our existing knowledge of pathogen reductions in dairy manure treatment methods. This study was conducted to enhance the understanding of human pathogen decay in liquid dairy manure in anaerobic (AN) and limited aerobic (LA) storage conditions. The decay of three pathogens (*Escherichia coli*, *Salmonella spp*., and *Listeria monocytogenes*) was assessed in bench-scale batch reactors fed with liquid slurry. A series of temperatures (30, 35, 42, and 50 °C) conditions were tested to determine the impacts of temperature on *Escherichia coli, Salmonella*, and *L. monocytogenes* decay in AN and LA conditions. Results showed prolonged survival of *E. coli* compared to *Salmonella* and *L. monocytogenes* in both LA and AN environments. Variations in survival among pathogens with temperature and environmental conditions (i.e., LA and AN) indicated the necessity of developing improved dairy manure waste treatment methods for controlling animal waste-borne pathogens. The results of this study will help in improving the current understanding of human pathogen decay in dairy manure for making informed decisions of animal manure treatment by stakeholders.

Keywords Dairy waste · Decay · Pathogens · Storage · Temperature

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Introduction

Increased disease outbreaks caused by pathogens over the last decades in the United States [1, 2] is a concern, and mitigating the public health risk requires controlling food and animal waste-borne pathogens in the environment because of potential interface between humans, animals, and the environment [3–5]. Many pathogens including *Escherichia coli* O157:H7, *Salmonella spp.* and *Listeria monocytogenes*, which persist in soil, manure, and water [6–9] has been linked to food related illnesses [10–12]. The shedding of these pathogens in cattle is reported extensively [13–17], and improved manure management has a potential to mitigate pathogens in the environment. In a flushed manure management system (widely used in large-scale dairy farms), water is used for flushing the manure in dairy barns, and flushed manure is passed through liquid—solid separator for separating solid materials, and liquid portion is stored in lagoons where occasional mixing (for partial aeration) is performed [18–20]. Both liquid and solid manure is applied into crop land as a fertilizer, which also poses risk of pathogen contamination in cropland [21–31].

Both the direct deposition of cattle manure to land and overland runoff events (especially after heavy rainfall) [30, 31] causes pathogens transport from crop land to streams resulting in increased surface and ground water contamination. An in vitro study by Bolton et al. [32] demonstrated

that when *E. coli* O157:H7 inoculated manure was applied to soil, the survival of *E. coli* O157:H7 in soil was prolonged up to 99 days after application. The survival of *E. coli* O157:H7 in bovine feces ranges from 49 to 126 days at 15 °C [33]. Multiple studies have suggested that manure amended agricultural field or contaminated irrigation water can be the potential source of pathogen contamination in agricultural products [34, 35]. Often raw food and vegetables are more vulnerable to indirect contamination by irrigation water or through soil treated with farm effluents [36]. Increased frequency of outbreaks of human infections associated with raw food and vegetables are attributed to many factors including lack of efficacy of sanitizers in killing pathogens of raw food and vegetables, an increase in per capita consumption of raw or minimally processed foods, increase in the number of immunocompromised consumers, and improved monitoring [37–39].

The survival of pathogens is influenced by many factors including pH, moisture content, C:N ratio, temperature, and solar radiation [40–42]. Temperature is reported to be a dominant factor affecting pathogen survival [36, 43]. Wang et al. [10] tested *E. coli* survival in bovine feces at 5, 22, and 37 °C by inoculating *E. coli* O157:H7 (3 [low] and 5 [high] orders of magnitude) in bovine feces. Authors reported *E. coli* survival in low and high inocula for 42 and 49 days at 37 °C and 49 and 56 days at 22 °C, respectively. At 5 °C, *E. coli* survived more than 60 and 70 days for low and high inocula. Depending on the temperature conditions, a prolonged survival of pathogens in manure and manure-amended soil is reported previously [44–47]. Relationships of *E. coli* O157:H7 infection with seasonal temperature pattern (the highest incidence occurred during the warmer months) are also reported [24, 47] indicating the change in pathogenicity or pathogen levels with temperature.

While manure is an important source of nutrients in soil, manure-borne pathogens have the potential to contaminate water as well as soil. If the manure is not treated properly prior to land application, manure-borne pathogens are likely to be transported from crop land to ambient water. Thus, it can potentially increase the risk of transmitting infectious agents such as *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* from crop land to ambient water. While many of the previous studies have focused on understanding of pathogen indicator survival in manure, this study was focused on studying the extent of human pathogen survival in liquid dairy manure under anaerobic (AN) and limited aerobic (LA) storage conditions. The objectives of the current study were to: (1) understand the effects of AN and LA environment on *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* survivals; and (2) assess the impacts of temperature and incubation period on the extent of the three pathogens decay during storage.

Materials and methods

Feedstock preparation

To prepare feedstock, solid dairy manure was collected from the University of California, Davis dairy facility located on the campus. The milking herd has a capacity of 100 cows and is registered with Holstein Association, USA. Fresh dairy samples were collected and refrigerated at −20 °C before starting the experiment. Prior to start the experiment, about 1.07 kg of dairy manure was thawed and mixed with 2L of deionized water. It was then sieved through a 850 μm (ASTM #20) mesh to separate the residue from the liquid slurry. The properties of solid manure (collected from dairy) and slurry (feedstock) samples are provided in Table 1.

Pathogen inoculum preparation

Pure strains of *E. coli* O157:H7 (ATCC# 35150) and *Salmonella typhimurium LT2* (ATCC # 700720) were cultured overnight in Luria–Bertani (LB) broth (Difco LB Broth Miller; Becton, Dickinson and Company, Sparks, MD, USA). The strains of *L. monocytogenes* (ATCC# BAA-679D-5) were cultured in Brain Heart Infusion (BHI) broth (BBL; Becton, Dickinson and Company, Sparks, MD, USA). A bench top incubator shaker (MaxQ 4000, Thermo Scientific, Ohio, USA) was used (100 rpm and 37 °C) for 24 h for growing the three pathogens. Quality control was ensured using a negative control in the respective growth media. The pathogen strains (ATCC#35150, ATCC # 700720, ATCC# BAA-679D-5) have been used previously to understand the pathogen survival in manure and food waste [48–51].

Feedstock inoculation with pathogens

The *E.coli* levels in manure before inoculation and after inoculation were verified by plating the pure culture samples in agar plates. MacConckey II agar with sorbitol (BBL, Becton, Dickinson and Company, Sparks, MD, USA), which is a selective and differential media for the detection of sorbitol-nonfermenting *E. coli* O157:H7, was used for *E. coli* testing and enumeration. When sample was tested on MacConckey II agar with sorbitol, the pure culture of *E.coli* O157:H7 produced colorless colonies. Pure strain of *Salmonella* Typhimurium LT2 was grown in Luria–Bertani (LB) broth, and subsequently pure culture

Table 1 Feedstock preparation and characteristics

Fresh dairy manure (g)	1065
Manure slurry (g) (after dilution of dairy manure with 2 L DI water and sieving)	2415
Moisture content of slurry (%)	97.4
Residue of diluted manure after sieving (g)	650
Moisture content of residue (%)	82.9
Initial pH of slurry	7.22
Initial concentration of *E.coli* in liquid slurry (CFU/mL) (before inoculation)	1.4×10^8
Initial concentration of *Salmonella* in liquid slurry (CFU/mL) (before inoculation)	0
Initial concentration of *L. monocytogenes* in liquid slurry (CFU/mL) (before inoculation)	0

was tested into Xylose Lysine Desoxycholate (XLD) agar (Difco, Becton, Dickinson and Company, Sparks, MD, USA) to verify the growth of *Salmonella*. Colonies with red-yellow with black centers in the agar plates were enumerated as *Salmonella*. The pure strain of *L. monocytogenes* grown in BHI growth media was tested in selective agar plates prior to mixing with the feedstock. Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aessulin-Mannitol (PALCAM) (HiMedia Laboratory, Mumbai, India) agar (with supplement) was used for plating *L. monocytogenes*. Gray-green colonies surrounded by dark brown to black halos in the agar plates were enumerated as *L. monocytogenes*. Pathogen enumeration in the feedstock from the experiment was conducted following the Bacteriological Analytical Manual (BAM) procedures [52]. To enumerate pathogens in feedstock, the slurry samples were serially diluted in Phosphate Buffer Solution (PBS), and the diluted samples (10^{-1}–10^{-6}) were plated in the respective agar for *E. coli, Salmonella*, and *L. monocytogenes* enumeration. The detection limit of pathogen was 10 CFU/mL of sample volume. All the samples were analyzed in duplicate.

Feedstock preparation

Pathogens were inoculated in feedstock. To inoculate feedstock with pathognes, the pure culture of *E. coli* O157:H7, *Salmonella, Listeria* were grown in 37 °C under controlled conditions. The *E. coli*, *Salmonella*, and *L. monocytogenes* levels in pure culture were 1.7×10^9 CFU/mL, 1.4×10^9 CFU/mL, and 1.7×10^9 CFU/mL, respectively. Subsequently, 4 mL of pure culture of each pathogen was centrifuged at 8000 rpm for 10 min to form the pellets of the three pathogens (*E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes*). Pellets were then dissolved in the feedstock (i.e., liquid slurry) and mixed with an overhead mixer (Carfamo Limited, Model BDC 250, Georgian Bluffs, Ontario, Canada) at 150 rpm for 10 min at room temperature to form a homogeneous mixture. The initial sample of the inoculated feedstock was collected and tested on the agar plates for calculating *E. coli*, *Salmonella*, and *L. monocytogenes* concentrations in the feedstock.

Experiment setup

The pathogen inoculated feedstock was filled into eight batch reactors (250 mL serum bottles). A schematic diagram showing the outline of experiment describing feedstock preparation and setup is shown in Fig. 1. The four reactors were LA (with no sealing cap), and other four reactors were AN (with sealing cap). Rubber septa were used to seal the AN reactors. The AN conditions in serum bottles were created using the procedure adopted by Pandey and Soupir [43] by escaping the head space air and creating a slightly negative head space pressure. In LA conditions, the top of the serum bottles remained open throughout the experiment allowing the air inside the reactors. Since this experiment was performed for simulating field environment, where aeration and mixing in lagoons are uncertain, the source of air in LA reactors was minimal mixing (i.e., continuous injection of air was not an option). All reactors (LA and AN) were then placed at four temperatures (30, 35, 42, and 50 °C). To understand the mixing effects, a minimal mixing was provided to both LA and AN reactors. Both LA and AN reactors were taken out of incubators every day (for 1 h) and placed on the top of a magnetic stirrer to provide mixing of feedstock inside the reactors. The samples from LA and AN reactors were collected (using the method of Pandey and Soupir [43]) for testing pathogens and calculating the change in pathogen concentrations. The experiment was conducted for 2 weeks. During the first 4 days, samples was collected twice (morning and evening), and afterwards sampling was performed daily. Since the main focus of this study was to assess pathogen decay, biogas including methane content of the biogas produced in AN reactors was not measured.

Data analysis

The decay processes of each pathogen in AN and LA storage conditions were analyzed for 13 days incubation period at four different temperatures. Based on the fact (observations) that the change in pathogen levels was limited in every single day, every other day samples (Day

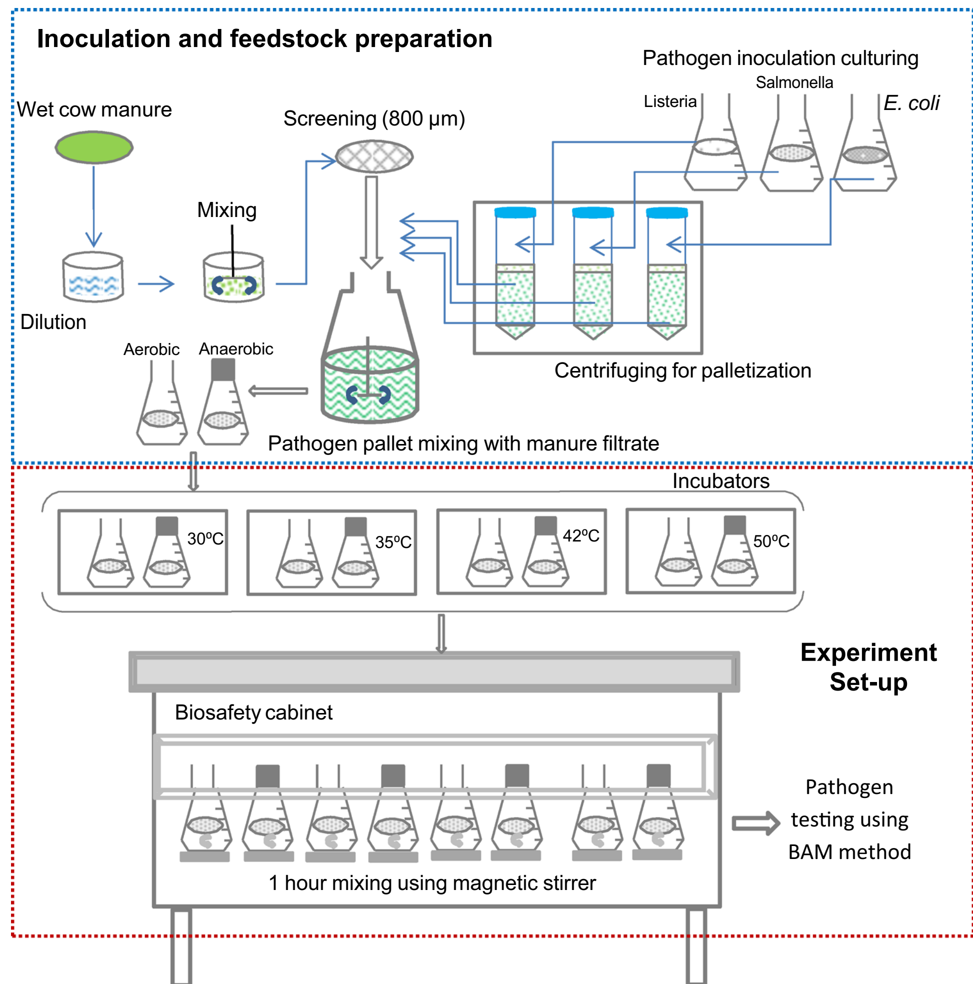

Fig. 1 Schematic diagram of pathogen inactivation experiment

2, 4, 6, 8, 10, 12 and 13) were used for statistical analysis. Firstly, analysis of variance (ANOVA) was performed to evaluate the impact of treatments (LA and AN) at four temperatures at different days of incubation. The data were log transformed before using the statistical models.

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is the pathogen enumeration at any particular day, μ is the overall mean, α_i is the effect of treatment *i*, β_j is the effect of temperature *j*, $(\alpha\beta)_{ij}$ is the effect of interaction between treatment and temperature, and ε_{ijk} is the random error.

Secondly, ANOVA was performed to evaluate the impacts of temperatures, and incubation days on pathogen levels where days were used as repeated measures.

$$Y_{jkl} = \mu + \gamma_k + \beta_j + (\beta\gamma)_{jk} + \varepsilon_{jkl} \quad (2)$$

where Y_{jkl} is the pathogen enumeration, μ is the overall mean, β_j is the effect of temperature *j*, γ_k is the effect of day *k*, $(\beta\gamma)_{jk}$ is the effect of interaction between temperature and day, and ε_{jkl} is the random error. Statistical analysis was done using PROC GLIMMIX in SAS [53]. An alpha level of $\alpha = 0.05$ was used to identify significant differences among treatments by "least significant difference" methods.

Results and discussion

The changes in *E. coli, Salmonella, and L. monocytogenes* levels at four temperatures under LA and AN storage conditions are shown in Figs. 2, 3, 4 and 5, respectively. Figure 2 shows changes in *E. coli*, *Salmonella* and *L. monocytogenes* in 30 °C. The changes in pathogens at 35 and 42 °C are shown in Figs. 3 and 4, respectively. Figure 5 shows the changes in pathogen concentrations at 50 °C. The initial level of *E.coli* in the feedstock was 5.4×10^7 CFU/mL after inoculating/spiking the slurry sample with strains. The initial levels of *Salmonella* and *L. monocytogenes* in the feedstock were 4×10^7 CFU/mL and 1.26×10^7 CFU/mL, respectively.

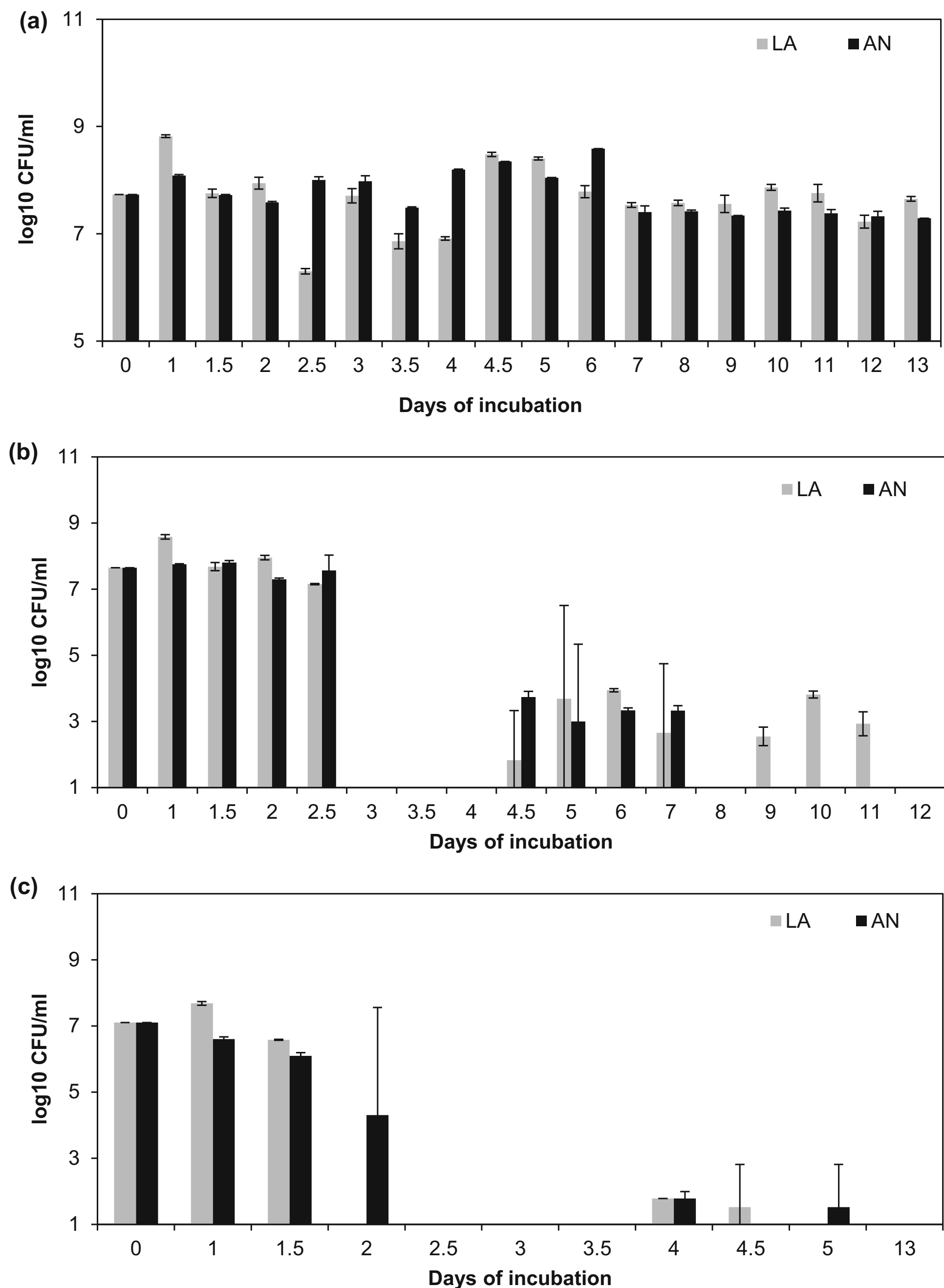

Fig. 2 Change in **a** *E. coli*, **b** *Salmonella*, and **c** *L. monocytogens* levels at 30 °C in limited aerobic and anaerobic conditions. *Gray bars* indicate limited aeration condition (LA), while *black bars* indicate anaerobic condition (AN). Standard deviaiton is show as *error bard*. *Bars* represent mean value of duplicate samples

The change in *E. coli*, *Salmonella* and *L. monocytogenes* over the time at 30 °C are shown in the Fig. 2a–c, respectively. There was an increase in pathogen concentration during the first sampling day (day 1) in both LA and AN conditions at 30 and 35 °C (Figs. 2, 3). Subsequently, a reduction in pathogen levels was observed. As shown in the Figures, the reduction in *E. coli* levels in both LA (minimal mixing condition) and AN reactors were considerably lower than *Salmonella* and *L. monocytogenes* in all temperature conditions (Figs. 2, 3, 4, 5). In the LA process at 30 °C, there was a 2.5 $\log_{10}$ reduction in concentrations of *E. coli* (Fig. 2a) during the first 3 days of experiment. The trend was similar in the LA and AN processes at 35 °C (Fig. 3a).

At 35 °C, the highest concentration (4.8×10^8 CFU/mL) of *E. coli* was observed during the fourth day of the experiment under LA condition, while it was highest (2.5×10^8 CFU/mL) on the sixth day of the experiment under AN condition (Fig. 3a). After the first week of the experiment, a decrease in concentration of *E. coli* was observed. An average of 3 $\log_{10}$ reduction of concentration was observed in LA condition, while there was a 1.9 $\log_{10}$

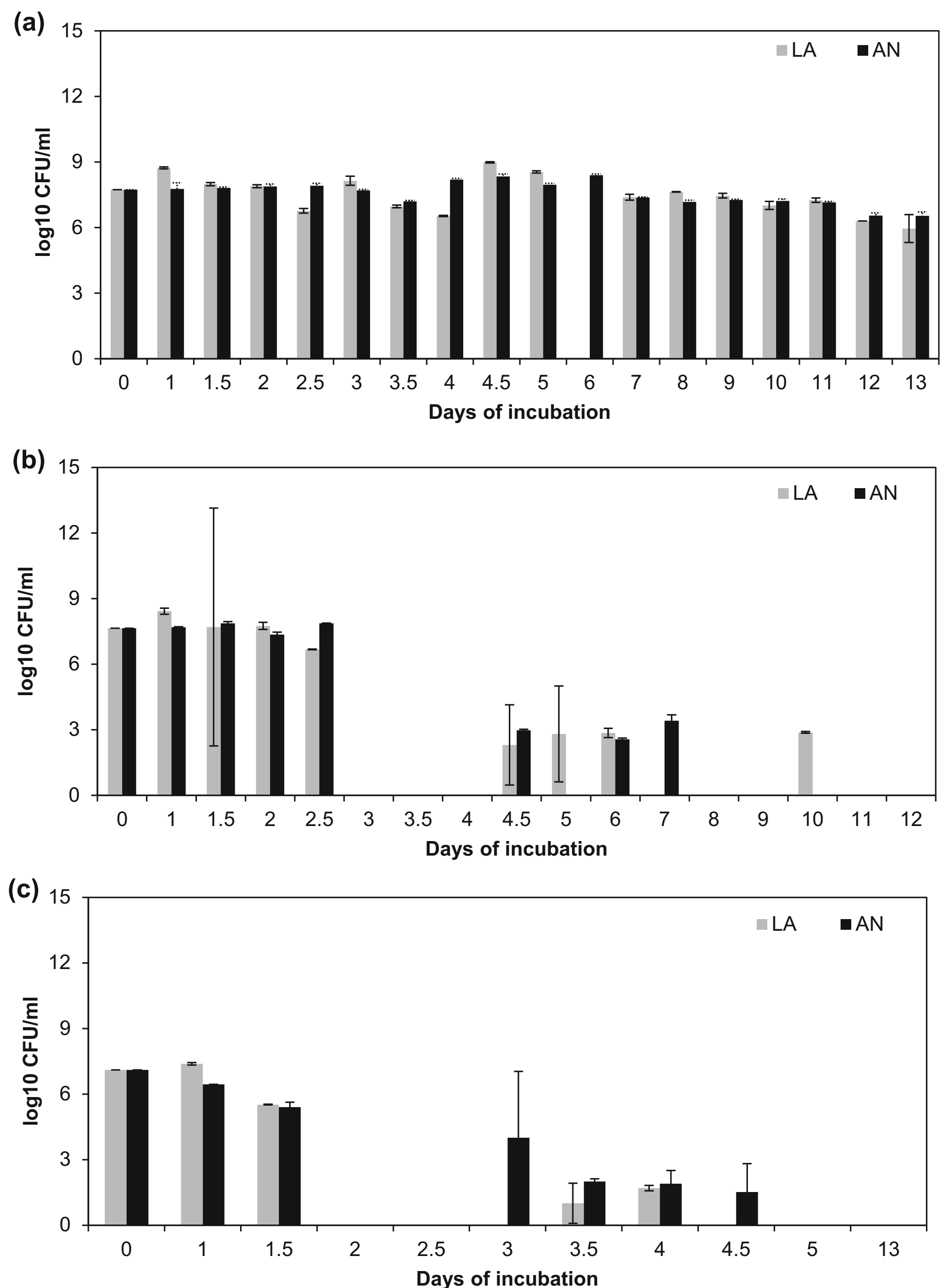

Fig. 3 Change in **a** *E. coli*, **b** *Salmonella*, and **c** *L. monocytogens* levels at 35 °C in limited aerobic and anaerobic conditions. *Gray bars* indicate limited aeration condition (LA), while *black bars* indicate anaerobic condition (AN). Standard deviaiton is shown as *error bard*. *Bars* represent mean value of duplicate samples

reduction in AN condition by the end of the experiment. At 42 °C, the initial concentration of *E. coli* went up to 2.8×10^8 CFU/mL and 5.5×10^7 CFU/mL, respectively, for the LA and AN processes (Fig. 4a) and followed the generic pattern of 30 and 35 °C. At a thermophilic temperature of 50 °C (Fig. 5a), there was a decrease in concentrations of *E. coli* in the LA and AN processes after the first day of the experiment. After 3 days of the experiment, 1.5 and 2.6 $\log_{10}$ *E. coli* reductions in the LA and AN conditions were observed. The concentrations went up to 3.2×10^8 CFU/mL and 3.3×10^7 CFU/mL during the next 2 days and consequently pathogen level was reduced. A similar study by Pandey and Soupir [26] observed considerably larger reduction in *E. coli* levels at 52.5 °C in 3.5 days of incubation in AN and aerobic conditions potentially due to continuous stirring and elevated temperature. While studying the decay of different *E. coli* species/serotype, another study by Smith et al. [54] reported similar variation in *E. coli* concentrations at 35 °C, but not at relatively higher temperature (55 °C).

Contrast to *E. coli*, the reduction in *Salmonella* levels was greater in all temperatures (Figs. 2b, 3b, 4b, 5b). As

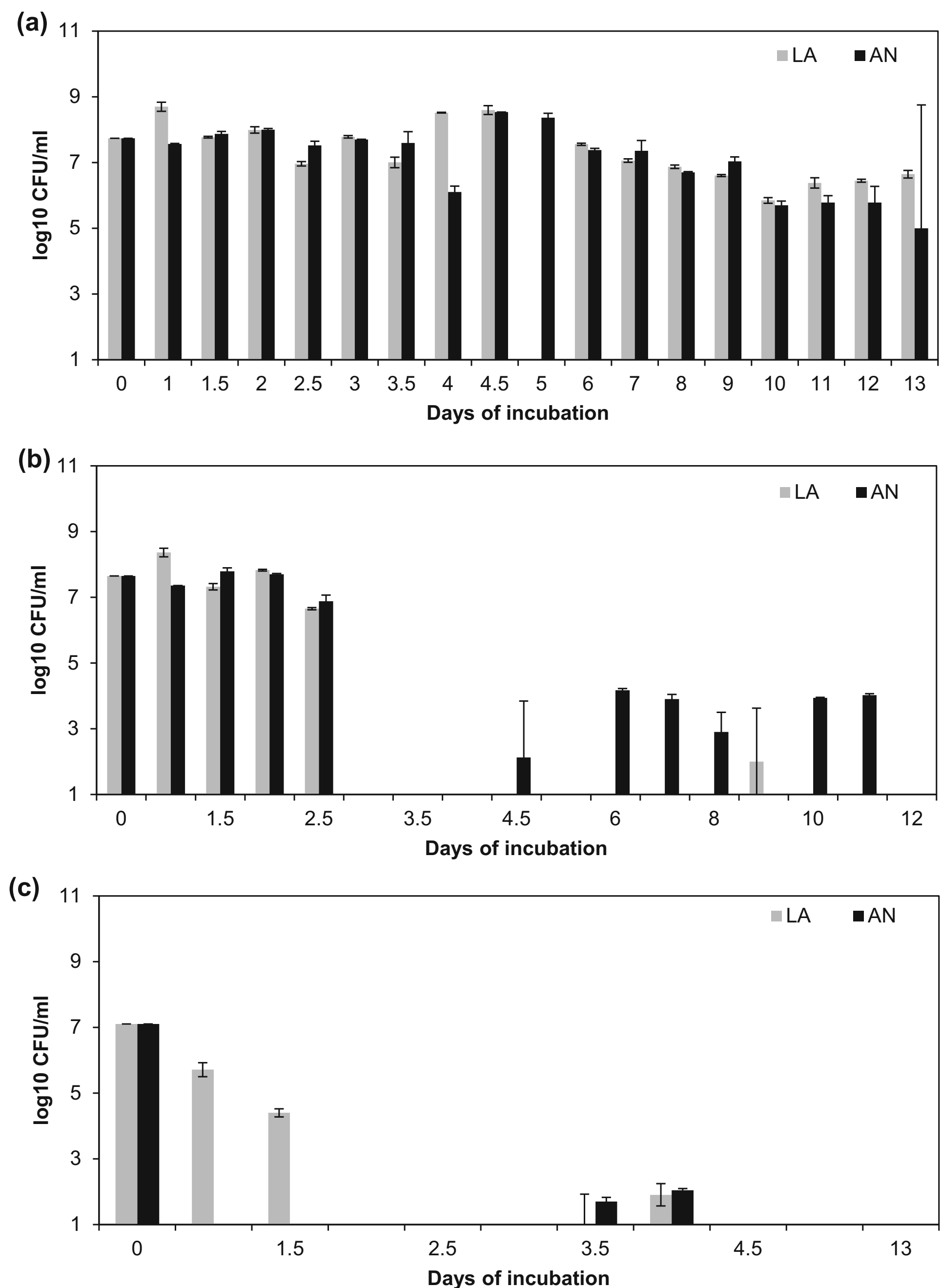

Fig. 4 Change in **a** *E. coli*, **b** *Salmonella*, and **c** *L. monocytogens* levels at 42 °C in limited aerobic and anaerobic conditions. *Gray bars* indicate limited aeration condition (LA), while *black bars* indicate anaerobic condition (AN). Standard deviaiton is show as *error bard*. *Bars* represent mean value of duplicate samples

shown in the figures, after 2 days of incubation, *Salmonella* level was undetectable (except 50 °C), although showed sporadically afterwards. There was a 4.7 $\log_{10}$ reduction from the initial concentration to the final day of detection (Day 11) in LA process at 30 °C (Fig. 2b) whereas a 4.3 $\log_{10}$ reduction at the end (Day 7) in AN process. At 35 °C, similar reductions in concentration (4.8 $\log_{10}$ and 4.2 $\log_{10}$, respectively) were observed from both LA and AN processes at the same time (Day 10 and Day 7, respectively) (Fig. 3b). At 42 °C, there was a 5.6 $\log_{10}$ reduction in LA condition, whereas there was a 3.6 $\log_{10}$ reduction in AN condition compared to the initial concentration (Fig. 4b). Similarly, at 50 °C, there was a 5.5 $\log_{10}$ reduction in LA process and 5.9 $\log_{10}$ reduction in AN process (Fig. 5b).

Erickson et al. [55] reported a 7.27 $\log_{10}$ *Salmonella* reduction within 4 days of aerobic composting of dairy manure with straw and cottonseed meal. While incubating the dairy manure at 37 °C, Toth et al. [56] found that *Salmonella* can survive for 2 weeks but it can persist longer at lower temperatures (22 and 4 °C). Another study by Semenov et al. [57] did not find any significant difference in survival of *Salmonella* (average decline 1.7 $\log_{10}$

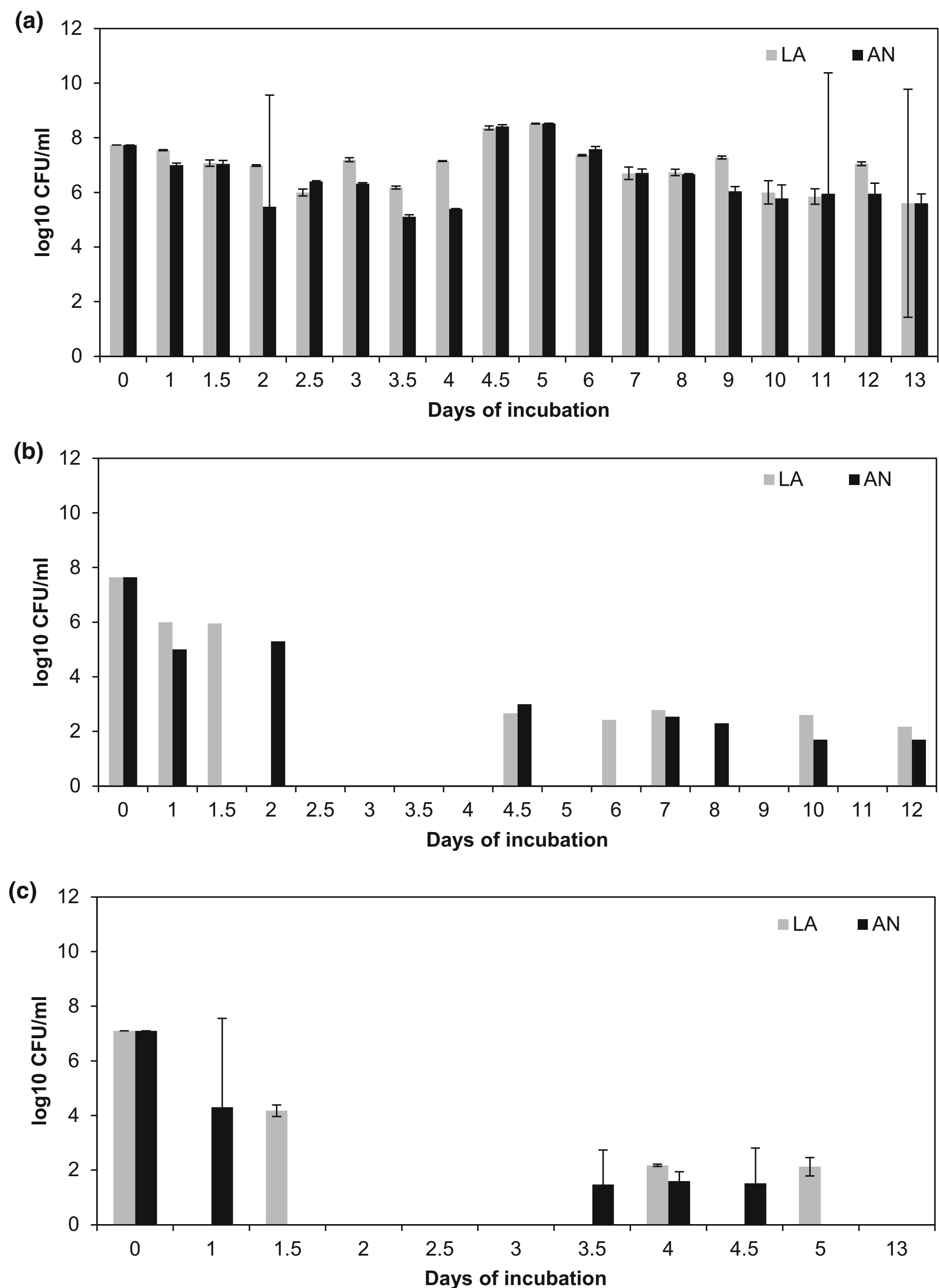

Fig. 5 Change in **a** *E. coli*, **b** *Salmonella*, and **c** *L. monocytogens* levels at 50 °C in limited aerobic and anaerobic conditions. *Gray bars* indicate limited aeration condition (LA), while *black bars* indicate anaerobic condition (AN). Standard deviaiton is show as *error bard*. *Bars* represent mean value of duplicate samples

cfu/gm after 5 days) between aerobic and AN storage of cattle manure during a 12 days incubation period.

Figures 2c, 3c, 4c, and 5c shows *L. monocytogenes* reduction pattern at 30, 35, 42 and 50 °C, respectively. Compare to *E. coli* and *Salmonella* reduction, the decay of *L. monocytogenes* was greater in all temperatures. Initial concentration of *L. monocytogenes* (1.26×10^7 CFU/mL) went to an undetectable level in 5 days of incubation at all four temperatures. In AN condition, the reductions in concentrations compared to initial concentrations were the same (5.6 $\log_{10}$) at 30 °C (Fig. 2c), 35 °C (Fig. 3c), and 50 °C (Fig. 5c) and 5.1 $\log_{10}$ at 42 °C (Fig. 4c). Erickson et al. [55] reported a 7.05 log10 *L. monocytogenes* reduction within 4 days after inoculation of dairy manure during the composting process. Other studies [58–61], however, reported relatively longer survival indicating considerable uncertainty in *L. monocyotenes* survival depending the environmental conditions.

Several previous studies focused on understanding of pathogen decay, especially in the AN process at different temperatures to achieve complete or partial pathogen decay [62–67] during anaerobic digestion process. In that

context, the current study is the first of its kind where the survivals of three pathogens were observed during the LA and AN conditions at four different temperatures in dairy manure slurry. Smith et al. [62] studied the decay process of *E. coli* NCTC 9001, *E. coli* O148 and *E. coli* O158 after inoculating with liquid raw sludge at three temperatures (35, 55, and 70 °C). They found an initial rise in *E. coli* concentrations at mesophilic temperature (35 °C), but not at the thermophilic range (55 and 70 °C). Aitken et al. [63] and Popat et al. [64] have studied initial temperature perturbation influence extensively. Pandey and Soupir [43] studied the *E. coli* decay kinetics in dairy manure at moderate (25 °C), mesophilic (37 °C) and thermophilic (52.5 °C) temperatures, and use the decay kinetics to derive the time—temperature—survival relationship for calculating *E. coli* survival in AN digestions. The focus of the current study was to understand the relative decay of *E. coli*, *Salmonella*, and *L. monocytogenes* in liquid dairy manure at mesophilic and thermophilic temperature conditions, and the results indicated a considerable difference in decay among the pathogens.

Similar to the results presented here, previous studies also reported inconsistence in the decay pattern of *E. coli* in cattle manure [43, 68]. Some of the *E. coli* serotype (generally *E.coil* O157:H7) has high tolerance against acidic and dry conditions and they can survive longer [10]. As an example, Kudva et al. [68] found that *E. coli* O157:H7 can survive for 47 days in aerated cattle manure piles. The results of this study suggest that *E. coli* survival in dairy manure was considerably longer than *Salmonella* and *L. monocytogens* in both AN and LA conditions indicating that the use of *E. coli* as an indicator for potential pathogen presence requires careful consideration. Overall, there was higher growth or survival of *E.coli* compared to *Salmonella* or *L. moncytogenes* at both treatment processes at all four temperatures. A study by Kim et al. [69] observed the differences in growth potential among bacteria species, and reported a greater growth of *E.coli* O157:H7 compared to *Salmonella* or *L. monocytognes* in dairy compost. Factors such as temperature as well as supply of oxygen are reported to be controlling factors for bacterial survival and growth in manure [58, 70].

To understand the impact of different temperatures on specific incubation day considering the treatments separately, ANOVA analysis of *E. coli* in Table 2 is showing the significant effects of temperature, day and temperature—day interaction on the prevalence of *E. coli*. Figure 6 shows interactions among temperature and incubation days in a three day interval of *E. coli* levels in LA and AN treatments separately. While evaluating the impact of treatments (LA vs AN) at each temperature on individual pathogens at a particular day, results of the statistical analysis showed that there was no significant interaction ($p = 0.114$) between treatment and temperature on Day 2 (for *E. coli* decay). There was a significant difference ($p = 0.01$) in concentration between the LA and AN treatments at 30 °C. Table S1 (supplementary data) shows a three way interaction among treatments, temperatures and days of incubation at $p < 0.0001$. Similar interaction was not noticed for other three temperatures (35, 42, and 50 °C). Unlike Day 2, there was a significant interaction ($p < 0.0001$) between treatment and temperature at Day 4 where the concentrations were significantly different between the treatment processes at 30, 35, and 42 °C. Further, there was a significant interaction ($p < 0.0001$) between treatment and temperature at Day 6 where the differences between treatments were only observed at 30 and 35 °C but not at higher temperatures (42 and 50 °C). The same trend was observed at Day 8. Significant interaction effects between treatment and temperature ($p < 0.0001$, $p = 0.014$, and $p < 0.0001$, respectively) was observed at 30 °C (Day 10), 50 °C (Day 12) and 30 °C (Day 13).

While comparing the *Salmonella* concentrations between the LA and AN conditions at four temperatures (Table S1), there was a significant interaction between treatment and temperature ($p = 0.004$) and the difference between the treatments were significant at 30 °C ($p = 0.001$) and 35 °C ($p = 0.007$) but not at 42 °C ($p = 0.108$) and 50 °C ($p = 0.983$) during Day 2. There was significant interaction effects between treatment and temperature at Day 6 and Day 10 ($p < 0.0001$) but not at Day 8 ($p = 0.275$). A significant difference in concentration was observed between the treatments at all four temperatures in Day 6. As shown in Figs. 2, 3, 4 and 5, frequent detection and non-detection of *Salmonella* was observed which could be due to re-growth of pathogen. A similar regrowth of *Salmonella spp.* in composted and stored biosolids have been reported previously by Russ and Yanko [71], Yeager and Ward [72], and Soares [73]. While assessing *L. monocytogenes* survival, we did not find significant interaction between treatment and temperature ($p = 0.07$) at Day 4 (except 50 °C). Since the concentrations of *L. moncytogenes* went to non-detection level at all four incubation temperatures during early phase of experiment, there was not enough data to run the analysis for all incubation days (Table S1).

As shown in Figs. 1, 2, 3, 4 and 5, both growth and inactivation patterns of *E. coli*, *Salmonella*, and *Listeria* were driven by temperatures. The ranges of *E. coli* levels over the 14 days incubation period at LA and AN conditions at 30 °C were $3.5 \times 10^8 - 0$, and $3.8 \times 10^8 - 1.1 \times 10^7$, respectively. The range of *Salmonella* levels over the 14 days incubation period at LA and AN conditions at 30 °C were $2.1 \times 10^8 - 0$, and $6.6 \times 10^7 - 0$, respectively. The ranges of *Listeria* levels for the same incubation period and temperature at LA and AN

Table 2 Analysis of variance (ANOVA) for *E. coli* enumeration considering aerobic and anaerobic treatments separately

Effect	Numerator DF	Denominator DF	*F* value	Pr > *F*
Limited aerobic (LA)				
Temperature	3	15	9.92	0.0007
Day	3	15	9.89	0.0008
Temperature × day	9	15	10.42	<0.0001
Anaerobic (AN)				
Temperature	3	15	3.11	0.0579
Day	3	15	12.6	0.0002
Temperature × day	9	15	2.63	0.0470

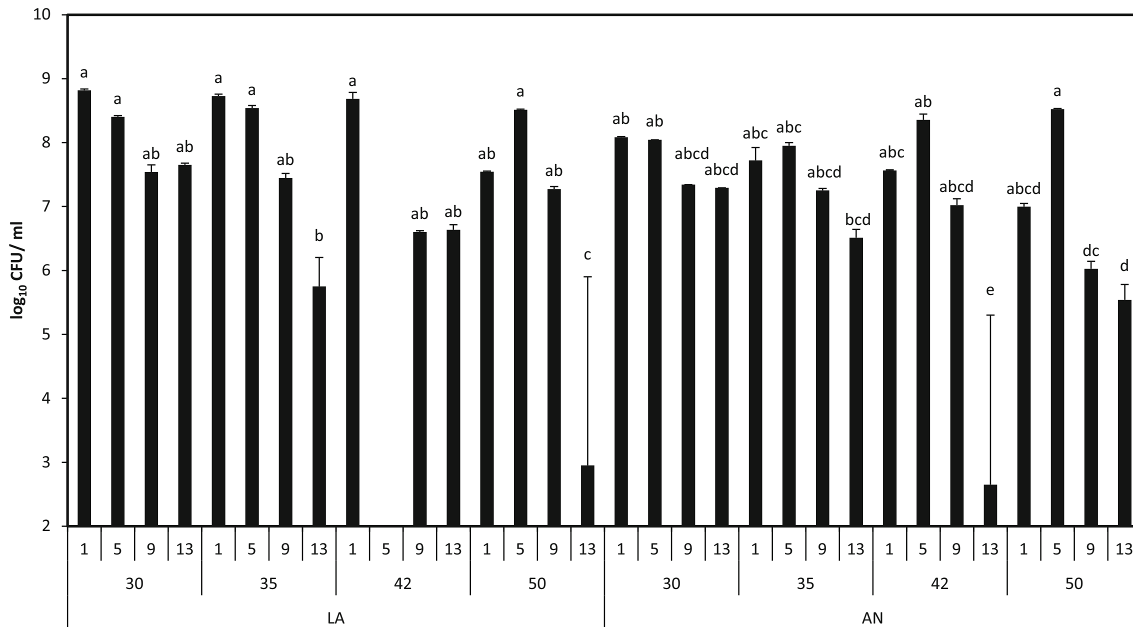

Fig. 6 *E. coli* levels at three days interval (Day 1, 5, 9, and 13) on four temperatures (30, 35, 42, and 50 °C) under two treatments (*LA* limited aeration and *AN* anaerobic) during the incubation period (*bars* with *different letters* are significantly different at $p < 0.05$)

conditions were $2.6 \times 10^7 - 0$, and $2.6 \times 10^6 - 0$, respectively. At higher temperature (50 °C), the range of *E. coli* levels over the 14 days incubation period at LA and AN conditions were $3.2 \times 10^8 - 0$, and $3.3 \times 10^8 - 0$, respectively. The ranges of *Salmonella* levels for the similar conditions were $7.5 \times 10^5 - 0$, and $1 \times 10^5 - 0$, respectively. The ranges of *Listeria* levels for the same conditions were $7.5 \times 10^3 - 0$, and $1 \times 10^4 - 0$, respectively. The pathogen survival time order (shorter to longer) was: *Listeria* < *Salmonella* < *E. coli*. This survival ranking was identical for all four temperatures (30, 35, 42, and 50 °C).

While assessing the impacts of temperature on pathogen survival, Himathongkham et al. [13] observed a first order rate of decay of *E. coli* 0157:H7 and *Salmonella* with periodic multiplication in solid manure and slurry at different temperatures (4, 20, and 37 °C). The quickest reduction was observed at highest temperature (37 °C). The decimal reduction time was 6–21 days in manure and 2–35 days in slurry. In addition to temperature, the moisture content of feedstock is also considered as one of the important factors influencing bacterial growth and survival in biosolids [72–75]. According to Kim et al. [76] about 20–30 % moisture content is required for pathogen growth in animal manure under laboratory conditions. In our study, moisture content of feedstock was >90 % throughout the experiment indicating that the moisture content was not an inhibiting factor in this study.

Conclusions

This study was designed to evaluate the performance of LA and AN storage conditions in decay of pathogens in dairy manure. The decay of three pathogens at four temperatures under minimal mixing (1 h/day) conditions was evaluated.

Results showed that the effects of both LA and AN conditions in pathogen reductions were almost similar in the minimal mixing condition potentially due to insufficient aeration of dairy manure. As continuous mixing is often cost prohibitive in dairy manure treatment at a large scale, infrequent mixing of stored flushed dairy manure is common, which may have limited impact on pathogen decay. *E. coli* survival was longer than *Salmonella* and *Listeria* in all temperature conditions. *Salmonella* and *L. monocytogenes* levels were reduced to non-detectable level in both LA and AN conditions within 3 days of incubation. Detection of these pathogens, however, was observed beyond 3 days potentially due to regrowth. We anticipate that this study will help in improving the existing animal waste treatment methods, and will support the stakeholders in making informed decisions while treating animal waste for pathogen reduction. Additional studies that focused on evaluating the regrowth of pathogens after treatment completion are needed to understand the recurrence of pathogens in large facilities treating manure where probability of cross-contamination is higher.

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