Clemson University
TigerPrints

All Dissertations | Dissertations

December 2020

Validation of Physically Heat-Treated Process for Poultry Litter and Analysis of Microbial Community of Animal Waste-Based Soil Amendments Using Sequencing Approach

Hongye Wang
Clemson University, hongyew@g.clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Recommended Citation

Wang, Hongye, "Validation of Physically Heat-Treated Process for Poultry Litter and Analysis of Microbial Community of Animal Waste-Based Soil Amendments Using Sequencing Approach" (2020). *All Dissertations*. 2727.
https://tigerprints.clemson.edu/all_dissertations/2727

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

VALIDATION OF PHYSICALLY HEAT-TREATED PROCESS FOR POULTRY LITTER AND ANALYSIS OF MICROBIAL COMMUNITY OF ANIMAL WASTE-BASED SOIL AMENDMENTS USING SEQUENCING APPROACH

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Food Science and Food Technology

---

by
Hongye Wang
December 2020

Accepted by:
Xiuping Jiang, Committee Chair
William C. Bridges Jr.
Annel K. Greene
Vijay Shankar
Tzuen-Rong Jeremy Tzeng

ABSTRACT

Biological soil amendments of animal origin (BSAAO), such as animal waste or animal waste-based composts, commonly used as organic fertilizer, may contain human pathogens such as *Salmonella* and *Listeria monocytogenes*. To reduce harmful microorganisms, animal waste can be treated by composting or other validated scientific methods. But insufficient treatment may introduce pathogens into agricultural fields. As a nutrient-rich fertilizer, poultry litter may also contain human pathogens with *Salmonella* spp. as a primary focus. Physical heat treatments can kill *Salmonella* in poultry litter with or without the composting process, but validation studies or guidelines are still needed for the litter processing industry to ensure the microbial safety of their products. Further, due to the ubiquitous nature of *L. monocytogenes*, it is essential to understand the ecology of this pathogen where it inhabits and then develop strategies to reduce *Listeria* contamination. We hypothesized that compost-adapted competitive exclusion (CE) microorganisms against *L. monocytogenes* exist in animal waste-based compost. In combination with the culturing method, the use of next-generation sequencing approaches is expected to speed up the discovery of those compost-borne CE microorganisms for controlling *L. monocytogenes* in pre-harvest environments. Therefore, the objectives of this study were to 1) test a nonpathogenic surrogate microorganism for validating desiccation-adapted *Salmonella* inactivation in physically heat-treated broiler litter, 2) validate the physical heat treatment of poultry litter composts using surrogate and indicator microorganisms for *Salmonella* in industrial settings, 3) use next-generation sequencing approaches to understand the microbial community profile and functions in animal waste-

based compost in the presence and absence of *L. monocytogenes*, and 4) isolate and identify the competitive exclusion microorganisms against *L. monocytogenes* in biological soil amendments.

In order to test a non-pathogenic surrogate for validating desiccation-adapted *Salmonella* inactivation in physically heat-treated broiler litter, thermal resistance of desiccation-adapted *S.* ser. Senftenberg 775/W was compared with that of *Enterococcus faecium* NRRL B-2354 in aged broiler litter. Samples of aged broiler litter with 20, 30, and 40% moisture content were inoculated separately with desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 at ca. 5 to 6 log CFU/g, and then heat-treated at 75, 85, and 150°C. At all tested temperatures, desiccation-adapted *E. faecium* NRRL B-2354 was more heat-resistant than desiccation-adapted *S.* Senftenberg 775/W ($P < 0.05$). During the treatments at 75 and 85°C, *E. faecium* NRRL B-2354 in aged broiler litter with all moisture contents was reduced by 2.9- to 4.1-log, and was above the detection limit of direct plating (1.3 log CFU/g), whereas *S.* Senftenberg 775/W could not be detected by enrichment (> 5-log reductions) during holding time at these temperatures. At 150°C, *E. faecium* NRRL B-2354 in aged broiler litter with 20 and 30% moisture contents was still detectable by enrichment after heat exposure for up to 15 min, whereas *S.* Senftenberg 775/W in aged broiler litter with all moisture contents could not be detected throughout the entire treatment. Our results revealed that *E. faecium* NRRL B-2354 can be used as a surrogate for *Salmonella* to validate the thermal processing of poultry litter by providing a sufficient safety margin. This study provides a practical tool for poultry litter processors to evaluate the effectiveness of their thermal processing.

Next, we used indicator and surrogate microorganisms for *Salmonella* to validate the processes for physically heat-treated poultry litter compost in both lab settings and commercial plants. Initial lab validation studies indicated that 1.2- to 2.7-log or more reductions of desiccation-adapted *E. faecium* NRRL B-2354 were equivalent to > 5-log reductions of desiccation-adapted *Salmonella* Senftenberg 775/W in poultry litter compost, depending on treatment conditions and compost types. Industrial plant validation studies were performed in one turkey litter processor and one laying hen litter processor. *E. faecium* was inoculated at ca.7-log CFU/g into the composted turkey litter and at ca. 5 log CFU/g into laying hen litter compost with respectively targeted moisture contents. The thermal processes in the two plants yielded reductions in *E. faecium* of 2.8 - > 6.4 log CFU/g (> 99.86%) of the inoculated. Similarly, for the processing control samples, reductions of presumptive indigenous enterococci were in the order of 1.8-3.7 log CFU/g (98.22% to 99.98 %) of the total naturally present. In contrast, there was less reduction of indigenous mesophiles (1.7-2.9 log CFU) and thermophiles (0.4-3.2 log CFU/g). Statistical analysis indicated that more indigenous enterococci were inactivated in the presence of higher moisture in the poultry litter compost. In conclusion, based on the data collected under the laboratory conditions, the processing conditions in both plants were adequate to reduce any potential *Salmonella* contamination of processed poultry litter material by at least 5-log, even though the processing conditions varied among trials and plants.

Further, to understand the complex interactions between native compost microorganisms and *L. monocytogenes*, compost samples collected across the US were subjected to the inoculation of *L. monocytogenes*, and then systematically analyzed using

16S rRNA gene, shotgun-metagenomic, and metatranscriptomic sequencing approaches along with culturing methods. The reductions of *L. monocytogenes* in dairy and poultry compost with 40 or 80% moisture content at room temperature after 72 h of incubation ranged from 0.1 to 1.1 log CFU/g. Regrowth of *L. monocytogenes* occurred in some compost samples after 72 h of incubation, ranging from 0.1 to 1.5 log CFU/g. The major bacterial phyla identified in all farms are *Firmicutes* (23%), *Proteobacteria* (23%), *Actinobacteria* (19%), *Chloroflexi* (13%), *Bacteroidetes* (12%), *Gemmatimonadetes* (2%), and *Acidobacteria* (2%). The statistical analysis of sequencing data revealed that microbial interactions were affected by environmental factors such as compost types and location, moisture levels and incubation length, rather than the inoculation of *L. monocytogenes*. Although the similarities percentage (SIMPER) results are not significant for all samples, some specific genera (*Bacillus*, *Sphaerobacter*, *Filomicrobium*, *Paucisalibacillus*, *Brumimicrobium*, *Steroidobacter Flavobacterium*, or *Chryseolinea*) were identified as discriminant microorganisms contributing to the variation in community composition due to the presence of *L. monocytogenes* on multiple farms. After 72 h of incubation, changes in the metabolic pathways and the increased abundance of the bacteriocins category in the compost samples containing *L. monocytogenes* suggest that the interactions between *L. monocytogenes* and compost microbiome may include competition for compost nutrients and the presence of antimicrobials produced by the compost microbiome. Findings from this study clearly indicated that microbial diversity and functional profiles were significantly ($P < 0.05$) affected by the compost source, compost stage, and collection farm. Furthermore, the presence of specific discriminant microbial species may suggest certain

compost samples as the potential sources for isolating CE microorganisms against *L. monocytogenes*.

Competitive exclusion (CE) microorganisms have shown great potential as environmentally friendly tools to control harmful microorganisms. In consideration of dairy and poultry compost containing a diversity of microbial species, it was hypothesized that the compost may be a good source for isolating compost-borne CE microorganisms, which can inhibit the growth of *Listeria monocytogenes*. In this study, CE strains were screened and isolated from compost using double- or triple-agar-layer methods. The addition of resuscitation promoting factor (Rpf) produced by *Micrococcus luteus* promoted the growth of slow-growing/viable but non-culturable species from compost. A total of 40 bacterial isolates were confirmed with anti-*L. monocytogenes* activities, and then tested for Gram-reaction, motility, biofilm-forming ability, and inhibitory spectra against produce outbreak-associated *L. monocytogenes* and surrogate strains, followed by identification via 16S rRNA gene sequencing. About 50% of the isolated CE strains were identified as *Bacillus* spp., and 17 of 40 isolates can inhibit more than 10 produce outbreak-associated *L. monocytogenes* strains, while 9 CE strains isolated from poultry litter compost were confirmed as motile and competitive biofilm formers. Those 40 CE isolates based on the origin of isolation were separated into two groups, i.e. poultry and dairy CE groups, and then tested for anti-*L. monocytogenes* activity in both compost extracts and the compost. After 168 h incubation, the growth potentials of *L. monocytogenes* were reduced by co-culturing with CE strains in compost extracts under all conditions by 0.1- to 1.9- log depending on incubation temperature, types, and ratio of the compost extracts. Results

showed that the inhibition effect from CE strains was higher in more concentrated compost extract (1:5) at 35°C or room temperature. In compost samples, the addition of CE strains can reduce *L. monocytogenes* population by ca. 1.2 log CFU/g at room temperature after 24 to 168 h incubation. The efficacy of CE strains was greater in the dairy compost as compared to that in the poultry litter compost. Findings from this study suggested that compost-adapted CE microorganisms have the potential as a biological control agent to control *L. monocytogenes* in agricultural environments.

In summary, current processes for physically heat-treated poultry litter in industrial settings have been validated, In addition, this study provided tools (surrogate and/or indicator microorganism for *Salmonella*, and custom-designed sampler) for litter processors to modify their existing process parameters to produce *Salmonella*-free physically heat-treated poultry litter, which can be used by the produce industry to grow microbiologically safe products. Both compositional and functional changes in microbial communities of compost samples were studied, and the CE microorganisms with antagonistic activities against *L. monocytogenes* were identified. Based on metagenomics and culturing approaches, we have demonstrated that composts can be a rich source of CE microorganisms as potential biological control agents, which can be used for foodborne pathogen control in both preharvest and postharvest environments. Results generated from this study have provided both validation and biological control tools for ensuring microbiological safety of animal waste-based biological soil amendments.

DEDICATION

To my beloved family.

First, I would like to dedicate this work to my parents, for their constant love and encouragement, for their sacrificing their life and time to encourage their only daughter to pursue her dream. Thank you for making the person who I am today. And my dear husband, Dr. Wenge Zhu, you are such a blessing in my life, thank you for your understanding, your support, and your love. This work would not have been possible without the support from you. Lastly, I want to special dedicate this work to my grandma, for being my awesome grandma in my life.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Dr. Xiuping Jiang, for her continuous guidance, patient assistance, and encouragement during my Ph.D. study. Her guidance helped me in all the time of my study and writing of this dissertation. Without the support from her, I would not have been able to complete this work. The advice and help from her will also shape my career greatly. My appreciation also goes to all my committee members, Dr. William C. Bridges, Dr. Annel K. Greene, Dr. Vijay Shankar, and Dr. Tzuen-Rong Jeremy Tzeng, for their time and sharing insightful suggestions during my Ph.D. pursuit. Apart from my dissertation committee, I am also wanting to thank our department staff, Ms. Kimberly Collins, Ms. Glenda Brown, Ms. Christina Boggs, and Ms. Jay Lyn Martin, for their help during my Clemson time. Finally, yet importantly, I would always thank my previous and current lab-mates for the time we spent together, of course, who are more than lab-mates.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

LITERATURE REVIEW

Biological soil amendments of animal origin

According to the U.S. Food & Drug administration Produce Safety Rule 21 CFR 112 (FDA, 2018); biological soil amendments of animal origin (BSAAO) contain materials in whole or partially from animal origin, such as animal by-products, manure, bone meal, feather meal and so on. In general, BSAAO can be treated by composting or some other validated scientific methods to reduce harmful microorganisms (Clements et al., 2019).

In 2017, there were 4,713 composting facilities in the U.S. Among those facilities, ca. 620 process multiple organics, which include feedstocks such as yard trimmings, food scraps, livestock manure, and industrial organics (BioCycle, 2017). It was estimated that 81.5% of the total number of organic produce farms use composted animal wastes (USDA, 2018). The total amount of animal manure produced in the U.S. was ca. 1.29 billion tons per year, including waste from poultry, swine, beef cattle, and dairy cattle (Gurtler et al., 2018). Owing to the high nutritional value for supporting plant growth, the treated or untreated animal waste can be used as organic fertilizer to enhance crop production and increase the soil fertility (Gascho et al., 2006). But the insufficient treatment or improper use of raw animal wastes or animal waste-based compost may introduce foodborne pathogens to produce production.

Prevalence of *Salmonella* spp. and *Listeria monocytogenes* in BSAAO

The list of human pathogens found in animal wastes or animal waste-based compost includes *Actinobacillus*, *Campylobacter*, *Clostridium*, Shiga toxin-producing *Escherichia*

coli (STEC), *Globicatella*, *Listeria monocytogenes*, *Mycobacterium*, *Salmonella* spp., *Staphylococcus*, *Streptococcus,* and so on (Larney et al., 2003b, Franke-Whittle et al., 2005, Grewal et al., 2006, Slana et al., 2011, Khan et al., 2016). From 2010 - 2017, 85 multistate outbreaks associated with fresh produce in the U.S. were attributed to STEC, *Salmonella* spp., and *L. monocytogenes*. *S. enterica* was linked to 67 % of these outbreaks, and *L. monocytogenes* was responsible for the most known deaths (67.3%) (Carstens et al., 2019). In fact, *Salmonella* spp. and *L. monocytogenes* are particularly important because of their low infectious dose and pathogenesis. Therefore, it is imperative to develop effective strategies to control *Salmonella* spp. and *L. monocytogenes* in animal waste or animal waste-based compost.

***Salmonella* spp. in BSAAO.** *Salmonella* are a group of Gram-negative, and non-spore forming rod-shaped bacteria. The genus *Salmonella* consists of two species, *S. enterica* and *S. bongori*, with the fact that *S. enterica* itself has over 2,500 serovars (Eng et al., 2015). According to the estimate by Centers for Disease Control and Prevention (CDC), *Salmonella* causes approximately 1.2 million illnesses and 450 deaths every year in the U.S. (CDC, 2016). Previous studies have reported that the *Salmonella* level ranged from 3 to 5 log/g in fresh livestock manure, including cattle, pig, poultry, and sheep (Hutchison et al., 2004ab). As compared to other pathogens, *Salmonella* is more frequently isolated from poultry litter or fecal samples and its prevalence level can range widely from 0 to 100% (Chinivasagam et al., 2010). As shown in Table 1.1, the prevalence of *Salmonella* in animal waste varies with the geographic location of the farms or types of animal waste. The contamination level of *Salmonella* varied among different animal waste, even on the same

farm. For example, *Salmonella* was most frequently isolated in broiler litter (55%), less frequently in swine manure (30%) and least frequently in dairy (13%) and beef (10%) manure, based on analysis of 2,523 manure samples collected in Canada (Flockhart et al., 2017). Effective composting practices can result in the elimination of *Salmonella* in poultry litter compost (Shepherd et al., 2010); however, the incidence of *Salmonella* was still observed after composting process due to the improper composting process or post-composting contaminations (Trimble et al., 2013).

Table 1.1 Prevalence of *Salmonella* and *L. monocytogenes* in animal waste or associated produce fields from 2000 to 2020.

Pathogens	Year/Location	Sample type	Sample size	Prevalence	References
Salmonella	2000-2001/Germany	Compost sample without indicating the animal type	24	20.8%	Hoszowski et al., 2001
	2002/ Republic of Ireland	Pig slurry	43	58.1%	Watabe et al., 2003
	2000-2002/UK	Cattle, pigs, poultry and sheep manure	1549	5.2% - 17.9 %	Hutchison et al., 2004b
	2002/Nigeria	Poultry fecal samples	120	38%	Orji et al., 2005
	2006-2007/Hungary	Broiler fecal samples	60	35%-43%	Nógrády et al., 2008
	N.A./US	Hen fecal samples	78	17%-56%	Li et al., 2007
	N.A./Nigeria	Layer litter samples	N.A.[a]	+[b]	Ngodigha et al., 2009
	2003-2005/US	Composted dairy manure	67	Culture negative for *Salmonella*	Edrington et al., 2009
	N.A./US	Broiler fecal samples	420	6%-39%	Alali et al., 2010
	N.A./Australia	Broiler litter samples	60	71%	Chinivasagam et al., 2010
	2004-2007/US	Samples of compost heaps with chicken litter or chicken carcasses	N.A.	26% surface and 6.1% internal samples (1st composting phase); absent in all samples (2nd composting phase)	Shepherd et al., 2010
	2008-2010/US	Chicken fecal samples	315	*Salmonella* was isolated from 29 farms (41.4%) with 65% prevalence in the 315 houses samples.	Donado-Godoy et al., 2012

	2007/US	Cow fecal samples	265	*Salmonella* isolate was recovered on 124 farms (46.8%)	Habing et al., 2012
	2013/US	Broiler compost	39	64%	Trimble et al., 2013
	2010-2011/US	Clinical and environmental samples from farm	204	Manure storage pile composites: 45% Wide turkey feces: 22.5% Soil: 10.3%	Jay-Russell et al., 2014
	N.A./ Brazil	Pig fecal sample	200	48.2%	Albino et al., 2014
	2016/US	Swine manure samples	130	*Salmonella* prevalence was 38.46% prevalence in swine manure,	Pornsukarom et al., 2016
	N.A./ Nigerian	Litter (n = 67) and feces sample (n = 75)	142	12.8% for litter sample, and 14.3 for feces sample	Fagbamila et al., 2017
	2006-2011/Canada	Fecal samples	2523	*Salmonella* prevalence: broiler manure (55%), swine manure (30%), dairy (13%) and beef (10%) manure.	Flockhart et al., 2017
	2017/ Burkina Faso	Poultry feces	103	52.42%	Kagambèga et al., 2018
	2013-2014/ Ethiopia	Poultry feces	549	4.7%	Eguale et al., 2018
	2016-2017/US	Solid bovine manure	91	15·4% for surface samples and 13·8% subsurface samples	Chen et al., 2019
L. monocytogenes	1999-2000/ US	Cabbage (n = 425), water (n = 205), and environmental sponge samples (n = 225)	855	3%	Prazak et al., 2002

N.A./ US	Field and potato samples	19	31.58%	Thunberg et al., 2002
UK/2000-2002	Livestock manure	1,549	29.8% and 30%, 19.0% and 19.8%, 29.2% and 44.4%, in fresh and stored cattle, pig, poultry, sheep manure, respectively.	Hutchison et al., 2005b
Ontario,Canada/2005-2007	Swine fecal samples	359	*L. monocytogenes* was only recovered from faces of weanling pigs and finisher pigs (3.3%)	Farzan et al., 2010
2009-2011/ US	Produce field	588	15%	Strawn et al., 2013a
2009-2011/ US	Fecal sample	61	15%	Strawn et al., 2013b
2010 / US	Terrestrial, water, and fecal samples in produce farm	124	42%	Weller et al., 2015
2010-2012/Canada	Hog manure (n = 9), irrigation water (n = 27), and muck soil (n = 288)	324	Not found in manure or soil, one positive from manure-contaminated irrigation water, and one positive from lettuce.	Guévremont et al., 2017
2014/ Malaysia	Composted animal manure and plant waste	60	8.3 – 16.70% from three different farms	Maurice et al., 2018
2016/ Jordan	Cattle feces	610	1.5%	Obaidat et al., 2019
2016-2017/ Iran	Wastewater effluent, sewage sludge and livestock manure	126	50% in sewage sludge, and 8% in manure sample	Gholipour et al., 2020

[a] N.A., not applicable;
[b] +, pathogen or selected microorganism was isolated.

***Listeria monocytogenes* in BSAAO.** *L. monocytogenes* is a Gram-positive, ubiquitous, and intracellular bacterial pathogen. *L. monocytogenes* has a multifactorial virulence system, with the thiol-activated hemolysin, listeriolysin O, which plays a crucial role in the organism's ability to multiply within host phagocytic cells and to spread from cell to cell **(**Farber et al., 1991). *L. monocytogenes* can cause listeriosis infections, which have a higher risk among certain groups of people, including elderly (> 65 years), pregnant women, and immune-compromised populations (Okike et al., 2013, Simon et al., 2018). In the U.S., *L. monocytogenes* is responsible for 19% of the total deaths due to the consumption of contaminated food, with fresh produce as an important source of contamination at both farm and processing environments (Scallan et al., 2011).

The occurrence of *L. monocytogenes* has been reported in both pre- and post-harvest environments, including fresh vegetables, soil, animal feces, and irrigation water (Guerra et al., 2001, Strawn et al., 2013ab, Gholipour et al., 2020). As shown in Table 1.1, the prevalence of *L. monocytogenes* in animal waste or associated produce fields in the last 20 years, from 2000 to 2020, ranged from 0 to 50%. The high level of *L. monocytogenes* was found in livestock manure and the manure-contaminated water, indicating that cross-contamination may occur. Strawn et al. (2013a) reported that runoff water from livestock farm was a contamination source for *L. monocytogenes* in irrigation water. *Listeria* spp. were more likely to be isolated from March to June according to Hutchison et al. (2005a), which revealed significant seasonal influences on the occurrence of *L. monocytogenes*. However, there were no confirmed correlations between the prevalence of *L. monocytogenes* and livestock diet (Hutchison et al., 2005ab).

L. monocytogenes is routinely isolated from both farm and processing environments, because it can follow and mediate a saprophyte-to-cytosolic-parasite transition by modulating the activity of a virulence regulatory protein called PrfA, using available carbon source (Freitag et al., 2009, de las Heras et al., 2011). Thus, *L. monocytogenes* can transit among animal waste, soil, water, fresh produce, and humans owing to this special life cycle (Zhu et al., 2017). The wide distribution of *L. monocytogenes* is also because of its ability to withstand extreme environmental conditions. The stress resistance mechanisms identified in *L. monocytogenes* are regulated by the alternative sigma factor (Chaturongakul et al., 2008). The ability of this pathogen to form biofilms can allow it to establish and persist for a long time in various environments. Therefore, fully understanding the survival characteristics of *L. monocytogenes* is essential to reducing food contamination with this pathogen.

The fate of *Salmonella* spp. and *L. monocytogenes* in animal waste or animal waste-based composts

In order to develop science-based control methods, it is important to identify factors that can significantly increase or decrease the likelihood of *Salmonella* spp. and *L. monocytogenes* survival in animal waste or animal waste-based composts. Thus, a systematic literature search was performed to identify the fate of *Salmonella* and *L. monocytogenes* in animal waste or animal waste-based compost. EBSCO (Academic Search Complete) and Web of Science were searched for peer-reviewed articles published between 2000-2020. The searching strings and study selection procedure were listed in

Table A1 and Figure A1, respectively (Appendix A). A total of 843 records were identified. After selection and quality assessment, 27 eligible studies were summarized in Table 1.2.

As shown in Table 1.2, the initial level of spiked pathogens ranged from 2 to 8 log cfu/g or ml, depending on the research purpose. Overall, factors that influenced the fate of *Salmonella* and *L. monocytogenes* in animal waste or animal waste-based compos can be grouped into following categories: i) Types and physical-chemical characteristics of animal waste; ii) Storage temperature; and iii) Background microbial community. Depending on these factors and experimental design, pathogens can survive < 1 to 405 days. Pathogens in animal waste or animal waste-amended soil can survive better in dairy manure, at a lower temperature, and with reduced background microbial load. Notably, most of the studies were carried out for evaluation of several factors together.

Table 1.2 Summary of reported studies on the factors affecting survival of *Salmonella* spp. and *L. monocytogenes* in animal waste and animal waste-based compost [a]

Pathogens	Matrix used	Initial levels	Treatment	Significant findings	Reference
Salmonella	Cow manure	6 log cfu/g	PA: *Salmonella* and *E. coli*; Temp: 4, 20, or 37°C; AWT: cow manure or cow manure slurry	The resulting decimal reduction times ranged from 6 days to 3 weeks in manure and from 2 days to 5 weeks in manure slurry; Decimal reduction times decreased with increasing in temperature.	Himathongkham et al., 1999a
	Chicken manure	6 log cfu/g	Water activity: 0.89–0.75	Highest death rate was found at intermediate a_w (0.89).	Himathongkham et al., 1999b
	Dairy manure compost	5 and 7 log cfu/g	AWT: soil amended with dairy cattle manure, poultry manure, or alkaline-pH stabilized manure, and using contaminated or not irrigation water.	Both contaminated manure compost and irrigation water can play an important role in contaminating soil and root vegetables with *Salmonella* for several months.	Islam et al., 2004a&b
	Hog manure slurry	4.5 log cfu/ml	Temp: 4, 25 or 37 °C; TD: 16 months: SE: fall, winter, spring	The decimal reduction time (DRT) of *Salmonella* in slurry stored at 37, 25 and 4°C ranged from 0.9 to 1.4 days, 8 to 19 days, and 22 to 60 days, respectively. Manure should be held for 60 days without commingling with fresh manure in reservoirs before application to fields, esp. for during winter.	Arrus et al., 2006

Cow manure	7 log cfu/g	AWT: cow manure and cow manure-amended soil; BMC: sterilized or non-sterilized soil.	*Salmonella* can survive in the manure-amended with sterilized soil for 405 days.	You et al., 2006
Bovine manure	*Salmonella*: 7.8 log cfu/g *E. coli*: 8.1 log cfu/g	PA: *Salmonella* and *E. coli*; Temp: 30 to 40°C; AWT: manure or manure slurry with different sampling depth.	Survival time of *Salmonella* reduced with increasing temperatures and amplitude in daily temperature oscillations.	Semenov et al., 2009
Pig manure	5 log cfu/g or ml	ST: solid and liquid fractions during storage	*Salmonella* can survive up to 112 days in pig manure under controlled storage conditions at 10.5°C.	McCarthy et al., 2015
Flushed dairy manure	4-5 log cfu/ml	PA: *E. coli* O157 and *Salmonella*	Aerobic system to be more effective than anaerobic system in terms of pathogen inactivation.	Pandey et al., 2016
Heat-treated poultry pellet amended soil extract	3 log cfu/g	BMC: unamended, nonsterile (UNS); unamended, sterile (US); amended, nonsterile (ANS); and amended, sterile (AS) TD: 96 h.	The regrowth of pathogen level order was: AS > ANS > US > UNS	Shah et al., 2019
Manure-amended soils	5 log cfu/g	Temp: 5, 21 or 37°C; AWT: poultry manure-amended clay loam or sand soil. TD: 6 weeks.	*Salmonella* reduced faster in sand soil. *Salmonella* reduced faster with higher temperature.	Phan-Thien et al., 2020
Fresh hog manure slurry	5 log cfu/g	MC: 60 – 80% SE: winter to summer (−18, 4, 10, 25°C), spring to summer (4,	Higher soil moisture, manure addition, and storage in the clay soil during winter increased *Salmonella* survival.	Holley et al., 2006

			10, 25, 30°C), or summer to winter (25, 10, 4, −18°C); TD: 180 days; ST: With or without clay soil.		
	Dairy lagoon and compost pile	7 log cfu/g	AWT: dairy lagoon, compost pile, and soil of a grass field. TD: year-round.	*S*. Newport survived in the static compost pile at 64°C for 18 h, whereas in the dairy effluent lagoon, the pathogen survived for >137 d.	Toth et al., 2011
	Dairy manure and poultry litter-based compost	5 or 2 log cfu/g	MC: 60 – 80% PA: *Salmonella* and *E. coli.* Temp: 5 and 22°C; AWT: dairy and poultry manure compost; ST: greenhouse condition.	Regardless of inoculum levels, *S. enterica* survived longer in hen manure-based compost compared with heat-treated poultry litter compost and low temperature can promote the extended survival of *S. enterica*.	Chen et al. 2018
	Cattle and wild animal feces	4 to 6 log cfu/g	AWT: wild animals (feral pigs, waterfowl, deer, and raccoons), from different states; TD: one year	*Salmonella* spp. can survive in the tested samples for up to 1 year, except in the sample collected from Ohio, in which *Salmonella* spp. was not detected after 120 days.	Topalcengiz et al., 2020
L. monocytogenes	Bovine manure-amended soil	5 to 6 log cfu/g	Temp: 5, 15 or 21°C; BMC: manure-amended autoclaved soil.	*L. monocytogenes* survived longer at lower temperature in the manure-amended autoclaved soil.	Jiang et al., 2004
	Pig manure	N.A.	Temp: 8 and 20°C; AWT: raw and biological treated manure; BMC: 81.5 – 94.8% and 67.8 – 79.2% VBNC cells.	*L. monocytogenes* increased more at 20°C. *L. monocytogenes* can enter VBNC state in the pig manure during storage. And the behavior of *L. monocytogenes*	Desneux et al., 2016

				was not influenced by the taxonomic composition of pig manure.	
	Dairy manure compost	7.4 log cfu/g	PA; *L. monocytogenes,* and *E. coli.* ST: Solid or liquid manure with different compost pile size.	*L. monocytogenes* can survive in solid manure pile for at least 29 weeks, compost pile size and temperature affect the pathogen survival.	Biswas et al., 2018
Salmonella and *L. monocytogenes*	Composted livestock manure or sewage sludge	5-6 log cfu/g	Temp: 50 °C; TD: 3 months; PA: *L. monocytogenes*, *Salmonella*; AWT: dairy cattle, beef cattle, pig, poultry layer, and sheep.	Pathogen survival time order (shorter to longer): *Salmonella*: dairy cattle < pig < poultry layer = sheep < beef cattle *L. monocytogenes:* dairy cattle = pig < poultry layer = sheep < beef cattle	Hutchison et al., 2004 & 2005
	Farmyard manure (FYD)	*E. coli* O157: 2.7-5.2 log cfu/ml, *Salmonella*: 3.2-4.5 log cfu/ml *L. monocytogenes*: 2.1-4.9 log cfu/ml *Campylobacter*: 2.1-4.2 log cfu/ml	PA: *E. coli* O157, *Salmonella*, *L. monocytogenes*, and *Campylobacter* AWT: dairy FYD, pig FYD, broiler liter, dairy slurry, and dirty water	Maximum pathogens survival period during storage (*Salmonella* and *L. monocytogenes* only). *Salmonella*: dairy FYD = pig FYD unturned = broiler litter < pig FYD turned < dairy slurry with 7% dry matter < dairy slurry with 2% dry matter. *L. monocytogenes*: dairy FYD = pig FYD (regardless turned or unturned) < broiler litter < dairy	Nicholson et al., 2005

			slurry with 7% dry matter < dairy slurry with 2% dry matter.	
Liquid swine manure and sawdust manure mix and dairy manure compost	6 log cfu/g	PA: *E. coli* O157, *Salmonella*, *L. monocytogenes*, and *Campylobacte*; ST: sawdust manure mix or untreated swine manure or pack storage; Temp: 25 to 55°C.	Both pathogens were unchanged in the sawdust manure mix and untreated liquid swine manure for up to 28 days at 25°C. For *Salmonella* and *L. monocytogenes*, both pathogens were destroyed most rapidly under thermophilic composting and persisted the longest in pack storage or low temperature composting.	Grewal et al., 2006 & 2007
Dairy compost extract	3 log cfu/ml	Temp: 22 to 35°C; PA: *L. monocytogenes*, *Salmonella*, *E. coli*; AWT: water extract of dairy compost of different ratios (1:2,1:5, and 1:10, w/v)	Indigenous microflora suppressed the pathogen regrowth in compost extract, especially at 35°C	Kim et al., 2009
Animal manure-based compost.	7 log cfu/g	Temp: 20 to 40°C; MC: 30 to 60%; PA: *Salmonella, L. monocytogenes,* and *E. coli*; AWT: dairy, chicken, and swine compost mixed with supplements.	Volatile acids promoted pathogen inactivation when temperatures are too low or quick heat lost at the surface of compost piles. Suboptimal MC (30-40%) were less effective for pathogen inactivation.	Erickson et al., 2014 & 2015
Dairy manure	7 log cfu/ml	Temp: 30, 35, 42, and 50 °C; ST: anaerobic (AN) and limited aerobic (LA);	Temp: Reduction of PA increased with higher temperature.	Biswas et al., 2016

		PA: *L. monocytogenes*, *Salmonella*, *E. coli*.	ST: Effects of both LA and AN condition in pathogen reductions were similar. PA: pathogen survival time order (shorter to longer) was: *L. monocytogenes* < *Salmonella* < *E. coli*

[a] Temp, Temperature; MC, Moisture content level; ST, Storage condition; PA, Pathogens; BMC, Background microbial community. SE, Season; TD, Testing duration. AWT, Animal waste types.

Types and physical-chemical characteristics of animal waste. The survival of pathogenic bacteria in different types of untreated animal waste or their amended soil has been studied under several factors such as moisture content (20 to 80%), water activity (0.89 to 0.75), and extra organic matter (2 to 7%) (Grewal et al., 2007, Wang et al., 2018, Phan-Thien et al., 2020). In general, the survival of *Salmonella* and *L. monocytogenes* varied considerably in different types of animal waste with different moisture contents, which was due to the microbial growth and metabolic processes which are determined by the moisture contents of animal waste. As revealed by multiple studies, *Salmonella* and *L. monocytogenes* can survive longer in dairy slurries, as compared to the other animal waste, such as pig, poultry, or sheep. Also, the addition of 2% dry matter (hay, straw or other bedding materials) can enhance the survival of these two pathogens (Hutchison et al., 2004&2005, Nicholson et al., 2005). As for the effects from physical-chemical characteristics of animal waste, survival time of pathogens was extended in the animal waste with suboptimal moisture contents (30% to 40% moisture contents). However, the death rate of *S. enterica* in animal manure had been restricted in a narrow range of water activity, in which the highest death rate was found in chicken manure with intermediate a_w (0.89) (Himathongkham et al., 1999a). Note that the types and characteristics of animal waste were not the only factors that affected the pathogens survival, as the storage conditions of animal waste also can affect the survival of both pathogens.

Storage conditions. Storage conditions can affect both biological and physio-chemical properties of animal manure, and therefore influence the survival of pathogens. Such conditions include surrounding temperature, storage format, aeration of compost and

the addition of dry matters during storage. In a study conducted by Biswas et al. (2018), *L. monocytogenes* survived in both the solid manure piles with periodic turning and in the slurry stored in small tanks for 29 weeks, whereas *E. coli* O157: H7 survived in the slurry sample for only 14 weeks. Survival time of pathogens was generally reduced with higher temperature. Arrus et al. (2006) reported that the decimal reduction time (DRT) of *Salmonella* in the slurry stored at 37, 25, and 4°C ranged from 0.9 to 1.4 days, 8 to 19 days, and 22 to 60 days, respectively, suggesting manure should be held for at least 60 days without adding fresh manure in reservoirs before application to fields, especially during winter. Pathogens in animal waste are exposed to the various environmental conditions, which affected the survival with some extends. Phan-Thien et al. (2020) reported that the persistence of *Salmonella* in poultry manure-amended soil was significantly affected by several factors, including temperature, soil type, types of poultry manure, and the interactions of these factors. Specifically, the addition of untreated poultry manure and lower temperature (5°C) can enhance the persistence of *S. enterica* in soil.

Background microbial community. The typical microorganisms presented in compost include *Alcaligenes faecalis*, *Arthrobacter*, *Brevibacillus*, *Enterobactericae*, *Bacillus* species, *Thermus* spp., *Streptomyces*, *Aspergillus fumigatus*, and *Basidiomyces* spp., which are bacteria, actinomycetes, or fungi (Rynk et al., 1992). Many studies reported that the fate of *Salmonella* and *L. monocytogenes* in animal manure and manure-amended soil ecosystem was affected by the composition of background microbial communities (Himathongkham et al., 1999b, Jiang et al., 2004, Desneux et al., 2016, Shah et al., 2019). In most cases, the reduced indigenous microbial load favored persistence of pathogens in

animal manure or manure-amended soil. For example, You et al. (2006) demonstrated that both multi-drug-resistant and drug-sensitive *Salmonella* had the longest survival (405 days) with the manure-amended sterilized soil, as compared to 184 and 332 days in manure and manure-amended nonsterile soil, respectively. The quick die-off of pathogens in nonsterile soil was mostly due to the antagonistic effects against either *Salmonella* or *L. monocytogenes* by the indigenous microflora. In contrast, Desneus et al. (2016) found that the behavior of *L. monocytogenes* was not influenced by the taxonomic composition of pig manure. The authors suspected that the *L. monocytogenes* had entered viable but non-culturable stage in the pig manure during storage. However, the modification on the indigenous microbial community, such as autoclaving or diluting, had omitted effects from the natural microbiota. As such, the complex interactions between the invasion pathogens and indigenous microflora still require further research.

Application of untreated animal waste may introduce potential microbial hazards to crop fields, thereby the NOP requires that raw animal manure should be incorporated into the soil more than 90 days prior to harvest for crops that have no direct contact with soil, and 120 days if the produce has direct contact with soil (FDA CFR, 2019). According to the Food Safety and Modernization Act (FSMA) Produce Safety Rule, raw manure must not contact produce during application and the potential for raw manure contact with produce after application should be minimized (FDA-FSMA, 2019). Sheng et al. (2019) conducted a 2-year field study to evaluate the impacts of dairy manure fertilizer application on the microbial safety of red raspberry. No Shiga toxin-producing *E. coli* (STEC) or *L. monocytogenes* was detected from fertilizer, soil, foliar, or raspberry fruit samples

throughout the sampling period for 2 years, whereas *Salmonella* in soil amended with contaminated fertilizer was reduced to undetectable level after 2 or 4 months of application. Because of amending agriculture soil with treated animal manure instead of fresh manure released less potential *Salmonella* and *Listeria* to the environment (Goberna et al., 2011), biological treatment options including composting (aerobic) and biogas (anaerobic) process can be used as pathogen control treatments to recycle animal waste back into the soil for crop use.

Composting process. Composting is a controlled biological process that broadly consists of four typical phases based on the temperature generated and active microbial community: mesophilic, thermophilic, cooling, and maturation phases (Figure 1.2). Normally, the composting process proceeds with solid or liquid materials within a moisture level range of 40 - 50% or 90 - 98%, respectively (USEPA, 2018). A variety of microorganisms, mesophilic, thermophilic, and thermo-tolerant bacteria, actinomycetes and fungi are actively involved in different phases to reach a satisfactory composting process (Hassen et al., 2001). As revealed in most composting studies, pathogens are killed primarily from the accumulation of heat (45 to 75 °C) generated by indigenous

microorganisms during the early phases of aerobic composting of animal manure (Lung et al., 2001, Larney et al., 2003a, Ceustermans et al., 2007).

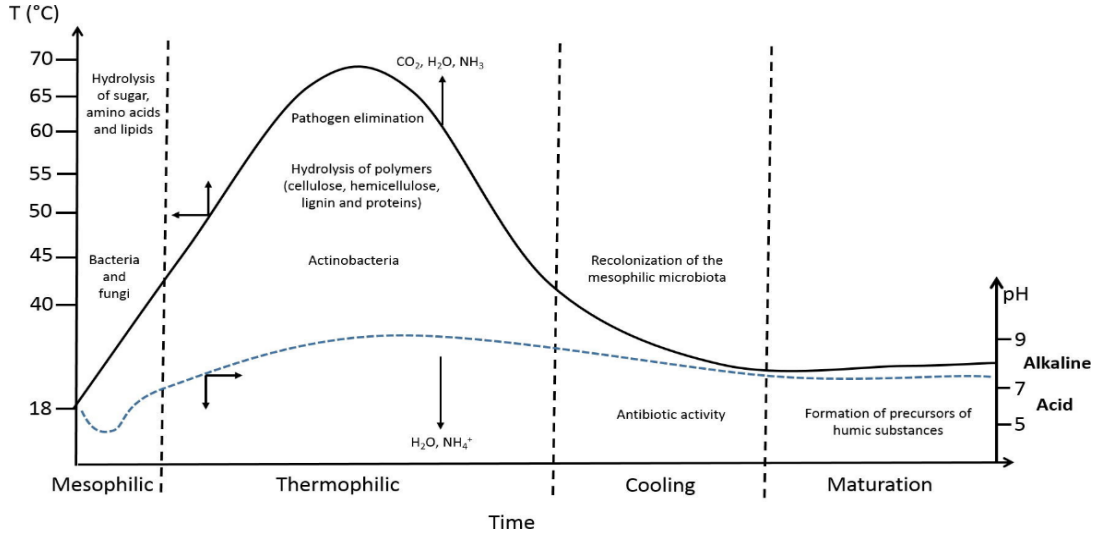


Figure 1.1 Composting process (Sánchez et al., 2017)

The finished compost should be thoroughly decomposed and pathogen-free. However, sporadic cases have reported the presence of foodborne pathogens in finished compost, indicating that finished compost made from animal waste are potential sources for pathogens (Miller et al., 2013, Dharmasena and Jiang 2018). These pathogens either survived the composting process or were cross-contaminated from raw manure and have potential for growth during the storage of compost. As specified by the FSMA produce safety rule, microbial standards for biological soil amendments of animal origin included less than 0.3 most probable number (MPN) per gram or milliliter of analytical portion for *E. coli* O157:H7, less than 3 MPN per 4 g or ml of total solids for *Salmonella* spp., and less than 1 cfu per 5 g or ml of analytical portion for *L. monocytogenes* (FDA, 2018). To achieve these standards, the FSMA Produce safety rule mandates the incorporation of alternative

treatments for reducing or eliminating human pathogens in raw animal waste before land application (Clements et al., 2019).

Physical heat-treatment strategies to inactivate pathogens in animal waste or animal waste-based compost

Physical heat-treatment of animal waste, such as pasteurization, and dry heating, can stabilize organic matter by reducing the moisture content of animal waste significantly after or without composting. In order to produce a pathogen-free final product, some critical factors, including time-temperature, moisture content of animal waste, heat sources, and monitoring/validation procedure, are needed to be controlled. Several organizations have provided time-temperature criteria on how to perform an effective heat treatment of animal waste (NOP, 2006, BioCycle, 2017). The minimal requirement for time-temperature is > 65°C for > 60 min to decontaminate fecal coliforms, *Salmonella*, and *E. coli* O157:H7 (NOP 2006). However, there is very limited research on the microbiological safety of these physically heat-treated animal waste products.

Table 1.3 summarizes laboratory-scale studies for evaluating the physical heat-treatment required to kill pathogens in animal waste or animal waste-based compost. The initial level of targeted microorganisms ranged from 2 to 10 log cfu/g, which represented a broad spectrum of pathogens existing in animal waste with various levels of heat resistance. Some key factors affecting the physically heat-treatment of animal waste are discussed below.

Table 1.3 Lab-scale physical heat treatments of animal waste with or without composting for pathogenic bacteria inactivation (Partially adapted from Zhen et al., 2017)

Animal waste	Tested microorganisms	Initial populations	Thermal-study set-up	Significant results	Reference
Dairy compost	*Clostridium difficile* endospores	5.5 log cfu/g	Samples were placed in enclosed Tyvek pouches and heated in an environmental chamber for dry heating source, and a water bath for moist heating source.	During holding time for 3 – 5 days, endospore counts reduced by < 68% at 55 and 65°C of dry heat treatment, whereas 80% - 84% and 75% – 99.9% reductions were observed at 55 and 65°C in wet heat treatment respectively.	Dharmasena et al., 2019
Aged chicken litter	Desiccation-adapted *Salmonella* spp.	7 log cfu/g	Samples were placed in an aluminum pan (i.d., 10 cm) and heated in an oven.	Desiccation-adapted *Salmonella* spp. reduced by 5-log in aged chicken litter with 20% moisture content required > 6 h of exposure at 70°C; and the exposure time required to achieve a 5 -log reduction in the number of cells gradually became shorter as temperature and moisture were increased. At 150°C, desiccation-adapted *Salmonella* spp. inactivated after 50 min in chicken litter with 20% moisture content.	Chen et al., 2013
Broiler chicken litter with different storage period	Desiccation-adapted *Salmonella* spp.	7 log cfu/g	Samples were placed in an aluminum pan (i.d., 10 cm) and heated in an oven.	At 150°C, desiccation-adapted *Salmonella* spp. inactivated after 45 min of heat treatment in 0-month litter, whereas for 3-, 6-, and 9-month litter	Chen et al., 2015

				samples, they could still be detected by enrichment for at least 1 h.	
Cow manure compost	*E. coli* and *Salmonella*	6 log cfu/g	Compost materials were placed into the drum rotation heat-treatment machine.	Both *E. coli* and *Salmonella* were not detected at 54–68°C for 6–24 h heat treatment.	Gong et al., 2007
Dairy compost (fresh and finished)	Acid-adapted *E. coli* O157:H7	7 log cfu/g	Samples were placed in enclosed Tyvek pouches and heated on the shelf of an environmental chamber with 70% humidity.	In fresh compost, acid-adapted and control *E. coli* O157:H7 survived for 19 and 17 days at 50°C, respectively, and 6 and 4 days for both types culture at 55°C and 60°C, respectively. Whereas the duration of survival for both cultures was shorter as compared to that in fresh compost.	Singh and Jiang 2012
Woodchip poultry litter, corncob poultry litter with Co_{60} woodchip or Co_{60}	*E. coli*, *S.* Pullorum, and *Arizona sp.*	4-5 log cfu/g	Samples were placed in a test thermal death tube (13×17 mm) and heated in a water bath.	*E. coli* inactivated at 68.3°C after 30 min in woodchip poultry litter with 18% moisture content. *S.* Pullorum inactivated at 62.8°C for 30 min and *Arizona sp.* inactivated at 57.2°C after 30 min in corncob poultry litter with 17% moisture content. *E. coli* inactivated at 57.2°C after 30 min in woodchip poultry litter with 39% moisture content. *E. coli* inactivated at 68.3°C after 30 min in both Co^{60} woodchip poultry litter with 29% moisture content and Co^{60} corncob poultry litter with 17% moisture content.	Messer et al., 1971

Broiler litter	Indigenous coliforms and total aerobic bacteria	>4.5 log cfu/g	Samples were heated in an oven or autoclave at 150°C for 10, 15, and 20 min at litter depth of 0.6 cm or at 121°C and steam pressure of 1.05 kg/cm^2 in the autoclave for 5, 10, 15, and 30 min at litter depth of 5.0 cm; at 150°C in the oven for 15 min at litter depth of 0.6 and 2.5 cm.	Coliforms were inactivated under all conditions. At 150°C, total aerobic bacteria were reduced to an undetected count in the oven after >20 min at litter depth of 0.6 cm. At 121°C in the autoclave for 10, 15, and 30 min at litter depth of 5.0 cm, and at 150°C in the oven for 15 min at litter depth of 0.6 and 2.5 cm.	Caswell et al., 1975
Dairy compost	Heat-shocked *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*.	6.81 – 7.33 log cfu/g	Samples were placed in enclosed Tyvek pouches and heated on the shelf of an environmental chamber.	At 50°C, it took 4 h for heat-shocked pathogens and control cultures to achieved 99% and 99.9% reduction. The heat-shocked pathogens have extended survival at lethal temperatures.	Singh et al., 2010
Broiler litter with pine shavings	*S.* Typhimurium	5 log cfu/g	Samples were placed in a perforated nursery flat (41.91 × 33.0 cm) and heated in a soil steamer cart for 5, 30, and 120 min.	*S.* Typhimurium was inactivated with steaming in 30 or 120 min.	Stringfellow et al., 2010
Broiler litter	*E. coli* and *S.* Typhimurium	8-9 log cfu/g	Samples were placed in a 50-ml plastic tube in a 100-mm diameter plastic container and heated in a water bath.	>99% inactivation was achieved for both pathogens at 55 and 65°C in 1 h.	Wilkinson et al., 2011

Aged broiler litter and fresh layer litter	*S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium	7 log cfu/g	Samples were placed in an aluminum pan (i.d., 10 cm) and heated in an oven.	*Salmonella* was inactivated in aged boiler litter with 30% moisture content at 70, 75, or 80°C after 300, 165, and 75 min, respectively. *Salmonella* was inactivated in fresh layer litter with 30% moisture content at 70, 75, or 80°C after 105, 90, and 60 min, respectively.	Kim et al., 2012
Poultry sludge	Fecal coliforms, *Salmonella* spp.	10.5 log MPN/g of total solids, 4.5 log MPN/g of total solids	Samples were heated in a 1.5-l hermetically closed thermal reactor.	Fecal coliforms and *Salmonella* spp. were both inactivated at 70°C in 120 min.	Ruiz-Espinoza et al., 2012
Layer manure	Total aerobic bacteria, yeast and mold, indigenous *E. coli*, and *Salmonella*	9.7, 3.4, and 7.4 log cfu/g, respectively	Samples were placed in a drying tray (1, 2, or 3 cm×10 cm×10 cm) and heated in an oven.	65.6-99.8% inactivation was achieved for total aerobic bacteria. 74.1-99.6% inactivation was achieved for both yeast and mold. 99.97% inactivation was achieved for *E. coli*. 100% inactivation was achieved for *Salmonella*.	Ghaly and Alhattab, 2013

Anaerobically digested dairy manure	Indigenous *E. coli*, *Salmonella*, and fecal coliforms	3.9, 3.8, and 2.2 log MPN/g, respectively	Samples were placed in a test tube and heated in a digestion unit with an aluminum block.	85 to 95% inactivation was achieved for fecal coliforms 87 to 96% inactivation was achieved for *E. coli*. 100% inactivation was achieved for *Salmonella*.	Collins et al., 2013
Dairy manure	*S.* Senftenberg, *Enterococcus faecalis*	6-7 log cfu/g, 6-7 log cfu/g,	Samples were placed in a tube and heated in a block thermostat.	*D*-values: 0.37 h at 55°C to 22.52 h at 46.0°C and 0.45 h at 55.0°C to 14.50 h at 47.5°C for *S.* Senftenberg and *E. faecalis*, respectively.	Elving et al., 2014
Ovine and bovine waste	*E. coli* O157:H7	6.5-7.0 log cfu/ml	Samples were placed in a sterile microcentrifuge tube and heated in a water bath at 50, 60, and 72°C for 10 min.	*E. coli* O157:H7 was inactivated at 60°C after 10 min.	Avery et al., 2009

Physiological status of pathogenic bacterial cells. Singh and Jiang (2012) reported the thermal resistance of pathogens in animal waste or animal waste compost was increased significantly if the cells were pre-adapted to stresses, such as heat-shock, acid-adaptation, or desiccation adaptation. Heat-shock can occur when the pathogenic cells are exposed to the sub-lethal temperature, i.e. during the mesophilic phase of the composting (Singh and Jiang, 2012). The stress response in the heat-adapted pathogens can promote the extended survival of pathogens in subsequent heat-treatment. For example, at 55°C, the heat-shocked cultures of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* are reduced for 1.2-, 1.9-, and 2.3-log within 1 h, respectively, whereas the corresponding control cultures had 4-, 5.6-, and 4.8-log reductions, respectively (Singh et al., 2010). The importance and frequency of desiccation stress requires special attention for the safety-production of heat-treated animal waste, as the moisture level can be reduced during the storage of raw animal waste, and at the surface of the compost pile, due to the evaporation or heating from a thermophilic process (Chen and Jiang 2017a). Studies have already demonstrated that desiccation-adapted *Salmonella* cells were more heat resistant than non-adapted cells in animal waste during physical heat treatment (Erickson et al., 2014, Chen et al., 2013), suggesting the need to use desiccation-adapted surrogate cells to validate the heat treatment conditions of animal waste to ensure a safety margin.

Types of animal waste Animal waste types and properties affect the survival profiles of microorganisms. As the chemical, physical, and microbiological properties changed along during the storage of poultry litter compost, the effectiveness of heat-treatment differed between fresh and aged chicken litters. Depending on initial moisture

contents of fresh chicken litter, a 7- log reduction of desiccation-adapted *Salmonella* required the heat-exposure of fresh chicken litter for 80.5 - 100.8, 78.4 - 93.1, and 44.1 - 63 min at 70, 75, and 80°C, respectively (Kim et al., 2012). As a comparison, in aged chicken litter with a 20% moisture content, a 5-log reduction of the desiccation-adapted *Salmonella* required > 360, > 360, and 240 to 300 min of exposure at 70, 75, and 80°C, respectively (Chen et al., 2013). Chen et al. (2015) further investigated the impact of chicken litter storage time and ammonia content on the thermal resistance of desiccation-adapted *Salmonella* spp., and reported that the thermal inactivation rates became lower in the chicken litter with extended storage time (6 months). Results from this study reported a significant ($P < 0.05$) loss of ammonia level in the chicken litter with longer storage time, indicating that the ammonia could be one significant factor influencing the thermal resistance of *Salmonella* cells in chicken litter.

Taken together, physical heat-treatment is a feasible and preferred strategy for inactivating pathogens in animal waste or animal waste-based compost. As a popular organic fertilizer, poultry litter is rich in nutrients and organic matter. But it is also a source of human pathogens with *Salmonella* spp. as the primary focus. To produce *Salmonella*-free products, validation studies on the *Salmonella* inactivation during physical heat treatment in industrial settings are urgently needed. However, the introduction of pathogens into the industrial setting is not recommended for plant validation studies. Hence, surrogate and indicator microorganisms should be used to understand the growth/survival behaviors of pathogens in industrial environments (Harris et al., 2013).

Surrogate microorganisms. Surrogate microorganism is a non-pathogenic strain that can respond to a specific treatment in a manner equivalent to a pathogenic strain (Hu et al., 2017). It can be used in validation studies in some industry settings. The systematic searching strings and selection procedure for studies that evaluated surrogate microorganisms for *Salmonella* spp. in physical heat-treatment processes were listed in Table A2 and Figure A2, respectively (Appendix A). A total of 232 records were identified, after selection and quality assessment, 33 eligible studies were summarized in Table 1.4.

Table 1.4 Surrogate bacteria evaluated for *Salmonella* spp. in physically heat-treatment studies since 2000.

Pathogens	Surrogates	Study matrix	Initial populations	Heat-treatment conditions	Suggestion on surrogate	References
S. Heidelberg, *S.* Typhimurium, *S.* Enteritidis, *S.* Infantis, and *S.* Hadar	Commercial *P. acidilactici* starter culture	Beef jerky	8 log cfu / strip for *Salmonella*, and 3.3 – 4.7 log cfu / strip for surrogate.	A wet-bulb spikes of 51.7°C for 60 min, 54.4°C for 60 min, 57.2°C for 60 min, 57.2°C for 30 min, and 60°C for 10 min., followed by drying at 48.9, 54.4, 60, and 76.7°C with hourly intervals.	Commercial *P. acidilactici* starter culture can be a potential surrogate.	Buege et al., 2006
S. Poona and *S.* Montevideo	15 nonpathogenic *E. coli*	Diluted bacterial suspension	Not describe	Samples with similar cell densities were heat treated at 60°C using a Techne submerged-coil heating apparatus.	5 out of 15 tested *E. coli* strains with less heat-resistance were recommended as *Salmonella* surrogates.	Eblen et al., 2005
S. Senftenberg 775W and *L. monocytogenes*	*E. faecium* NRRL B-2354, *P. parvulus* HP, and *P. acidilactici* LP	Ground beef	7.5 log cfu/g	Samples were heated in a circulating water bath at 58, 62, 65, or 68°C.	*S.* Senftenberg 775W was the most heat-resistant pathogens, however, thermal treatments conditions that can kill *E. faecium* NRRL B-2354 will also kill both *Salmonella* and *L. monocytogenes*.	Ma et al., 2007
S. Enteritidis	*E. coli* K12	Liquid egg	7.5 log cfu/ml	Samples were placed inside a sealed aluminum thermal-death-time disk and heat-treated in a water bath at 52 to 60°C.	Non-pathogenic *E. coli* K12 may serve as a surrogate for pathogenic *S.* Enteritidis in liquid egg	Jin et al., 2008

					during thermal pasteurization.	
S. Enteritidis	Commercial lactic acid bacteria	Ground-and-formed beef jerky	8 log cfu per beef strip	Samples were run in a dehydrator at 68.3°C for 0, 3.5, and 7 h.	Commercial lactic acid bacteria (LAB) can be used as *Salmonella* surrogate for validating beef jerky process.	Borowski et al., 2009
S. Enteritidis PT 30	*E. faecium* NRRL B-2354	Almonds	Not describe	Samples were heat treated in a custom built, computer-controlled, laboratory-scale, moist-air convection oven at 121 to 204°C for targeting 5-log reduction.	*E. faecium* NRRL B-2354 can be used as a conservative surrogate for SE PT30 during moist-air heating, and this organism is also likely to be an acceptable surrogate for steam heating.	Jeong et al., 2011
Salmonella	A surrogate cocktail combination of *E. faecium* ATCC 8459 and *E. faecium* ATCC 35667	Peanut	6.5 to 7.7-log cfu/g	Peanut dry roasting process was performed in industrial setting, with temperatures (149, 163, 177, and 191°C).	Roasted peanut process has been validated using surrogate microorganisms.	Poirier et al., 2014
S. Typhimurium, *S.* Infantis, and *S.* Hadar	*P. acidilactici* and *S. carnosus*	Frankfurter batter and ground beef	7 log cfu/g	Samples were placed into bags inside a wire-mesh basket and were heated in a preheated circulating water bath at 55°C for up to 120 min.	Due to the less heat resistance of surrogates as compared to the targeted pathogens, the use of *P. acidilactici* and *S. carnosus* as *Salmonella* surrogate to validate thermal processing	Vasan et al., 2014

					interventions in ground beef and frankfurter batter is not recommended.	
S. enterica	*E. faecium* NRRL B-2354	Carbohydrate-protein meal	6 log cfu/g	Samples were extruded in a model 2003 GR-8 single-screw extruder at 50 – 100°C for 5- log reduction.	*E. faecium* can provide enough safety margin of error for eliminating *Salmonella.*	Bianchini et al., 2014
S. Tennessee	*E. faecium* NRRL B-2354	Talc powder	8 – 9 log cfu/g; using dry inoculum	Samples were sealed into a vacuum bag, and heat treated in a controlled water bath at 85°C.	*E. faecium* can be a conservative surrogate for *S.* Tennessee.	Enache et al., 2015
Salmonella serovars	*P. acidilactici* ATCC 8042 and *E. faecium* NRRL B-2354	Dry pet foods	Not describe	Samples were heated in a circulating water bath between 76.7 and 87.8°C.	*P. acidilactici* ATCC 8042 can be utilized as a surrogate for *Salmonella* in dry pet food at < 90°C.	Ceylan et al., 2015
S. Senftenberg, *S.* Typhimurium, and *S.* Newport	*E. faecium* NRRL B-2354 and *Saccharomyces cerevisiae*	Hamburger buns	8 log cfu/g	Samples were baked for 13 min in a conventional oven (218.3°C), with internal crumb temperature increasing to ~100°C in 8 min of baking until removal from the oven.	*E. faecium* demonstrated greater thermal resistance compared with *Salmonella*, not *S. cerevisiae.*	Channaiah et al., 2016
S. Typhimurium PT 42	*E. coli* 3A-I, *E. faecium* 2B-I, and ten different isolates from flour.	Flour	5 log cfu/g	Samples were placed into 2 ml screw top glass vials, and heat treated in agitation water bath at (70, 75, and 80°C) for 12 to 60 min.	*Pantoea dispersa* strain JFS isolated from flour was suggested as a surrogate for *S.* Typhimurium PT 42.	Fudge et al., 2016

S. Typhimurium	Avirulent strain of *S.* Typhimurium	Diluted bacterial suspension	~ 7 log cfu/ml	Bacterial cultures were heat-treated on a water batch at 50°C for up to 10 min.	The avirulent strain of *S.* Typhimurium contains no pathogenicity islands, and shared similar thermal resistance with that of the wild type.	de Moraes et al., 2016
S. Senftenberg 775W, *S.* Typhimurium, *S.* Anatum, *S.* Montevideo, *S.* Tennessee, and *L. monocytogenes*	*E. faecium* NRRL B-2354	Low moisture foods	7 – 8 log cfu/g	Samples were placed into thermal cells and heat-treated in an oil bath. at four temperatures between 70 and 140°C.	*E. faecium* NRRL B-2354 can be a suitable surrogate for *Salmonella* for low moisture foods, but not for the sugar containing confectionery formulation	Rachon et al., 2016
Salmonella	*Cronobacter sakazakii*, and *Pediococcus acidilactici*	Galactooligosaccharide	*Salmonella* (5.1 to 5.8 log cfu/g) or *C. sakazakii* (5.3 to 5.9 log cfu/g) and *P. acidilactici* (6.1 to 6.5 log cfu/g)	Samples were heat treated at 70 – 85°C to determine D- and Z- values.	*P. acidilactici* had higher D-values than did *Salmonella* and *C. sakazakii*, which can be used as conserved surrogate for *Salmonella*.	Bang et al., 2017
S. enteritidis PT 30	*E. coli* ATCC 25922	Almond powder	~ 3 log cfu/g	Samples were treated by a controlled atmosphere/heating block system at four temperatures between 65 and 80°C under different gas concentrations and heating rates.	No directly comparison of thermal resistance between *Salmonella* and *E. coli* was provided in this study.	Cheng et al., 2017

S. Enteritidis PT30	*E. faecium* NRRL B-2354	Almond kernels	~ 8 log cfu/g	Samples were heated in a pilot-scale, moist-air impingement oven at 121, 149, or 177°C.	The surrogate yielded lethality prediction that were statistically like *Salmonella.*	Jeong et al., 2017
S. Enteritidis PT30	*E. faecium* NRRL B-2354	Flaxseed, sunflower seeds, and peppercorn	~ 7 - 8 log cfu/g	Samples were heat treated with vacuum steam 75, 85, 95, and 105°C.	*E. faecium* can be used as surrogate for *Salmonella* PT 30 for vacuum steam pasteurization.	Shah et al., 2017
S. Enteritidis	*E. faecium* NRRL B-2354	Wheat flour	~ 8 log cfu/g	Samples were heated treated at 75, 80, and 85°C in sealed aluminum-test-cells.	*E. faecium* can be a conservative surrogate for *Salmonella* in thermal processing of wheat flour.	Liu et al., 2018a
S. Enteritidis	*E. faecium* NRRL B-2354	SiO_2 particles	7.8 log cfu/g	Samples were heated in ethylene glycol oil bath at 80°C.	The thermal resistance of both *S.* Enteritidis and its surrogate increased with decreasing relative humidity in the testing environments.	Liu et al., 2018b
Salmonella	*E. faecium* NRRL B-2354	Oat flour	7 - 8 log cfu/g	Samples were heat treated in a lab-scale single-screw extruder running at different screw speeds (75 to 225 rpm) and different temperatures (75, 85, and 95°C).	Effect of different product and process parameters on inactivation of *E. faecium* NRRL B-2354 was different from that of *Salmonella,* suggesting another surrogate may be	Verma et al., 2018

					preferred and needs to be identified.	
S. Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Newport, and *S.* Choleraesuis.	Non-pathogenic *E. coli* surrogates from ATCC	Ground beef	6 log cfu/g	Samples were plated into sample bags and heat-treated in a water bath at 54, 57, 60, and 63°C.	*E. coli* surrogates can be used as *Salmonella* surrogate in ground beef.	Redemann et al., 2018
S. enterica and *L. monocytogenes*	*E. faecium*	In-shell pecans	7.88, 7.58, and 6.53 cfu/g for *Salmonella*, *L.monocytogenes*, and *E. faecium*, respectively.	In-shell pecans were subjected to hot water at 70, 80 or 90°C for 1, 2, 3, 4 or 5 min.	Non-pathogenic *E. faecium* showed similar resistance to hot water as *S. enterica. L. monocytogenes* was the least heat resistant pathogen to hot water treatment.	Kharel et al., 2018
Salmonella	*E. faecium* NRRL-B2354	Almond meal	~ 8 log cfu/g	Samples were kept in aluminum test cells and submerged into a water bath for heat treatment at 80°C.	*E. faecium* NRRL-B2354 was more heat resistant in dry inoculation methods, as compared to the wet inoculation.	Ahmad et al., 2019
S. Enteritidis PT30	*E. faecium* NRRL-B2354	Peanuts and pecans.	7.5 to 8.7 log cfu/g	Peanut kernels were heat treated by dry air heating conditions, (120°C (20, 30, 40 min), 130°C (10, 20, 30 min), 140°C (10, 20, 30 min)); Pecan kernels were heat treated by Oil heating conditions (116°C, 121°C,	*E. faecium* is a valid surrogate for peanut stagnant dry heat and pecan conditioning and oil heating treatments, but not for dry air heating of peanuts.	Brar et al., 2019

				and 127°C for 0.5, 1.0, 1.5, 2.0, 2.5 min). Water heating conditions (75°C (20, 40, 80, 120 s), 80°C (20, 40, 80, 120 s), 85°C (20, 40, 80, 120 s), 90°C (20, 40, 60, 80 s), and 95°C (20, 40, 60, 80 s) were evaluated for inshell pecans.		
Salmonella serovars (Enteritidis, Agona, Typhimurium, Tennessee, and Newport)	*P. acidilactici* ATCC 8042 and *E. faecium* NRRL-B2354	Toasted oats cereal and peanut butter	6 log cfu/g	Samples were put into stomacher bags, and treated in a water bath at 80, 85, 90, and 95°C for toasted oats cereal, and 63, 68, 73, and 77°C for peanut butter.	*P. acidilactici* had the similar heat tolerant as *E. faecium*, however, *P. acidilactici* can only be used as *Salmonella* surrogate in toasted oats cereal when heat treated at > 85°C.	Deen et al., 2019
S. Typhimurium	*E. faecium* NRRL-B2354	Cocoa powder /	~ 8 log cfu/g	Cocoa powder (a_w = 0.3 or 0.4) was heat treated in an ethylene glycol bath at 70–80°C.	The suitability of *E. faecium* as a surrogate for *Salmonella* varies as a result of aw of cocoa powder.	Tsai et al., 2019
S. Senftenberg 775W, *S.* Enteritidis PT 30, *S.* Montevideo and *L. monocytogenes*	4 non-pathogenic surrogates *E. faecium*, *E. coli* P1, *E. coli* K12 and *L. innocua*	Black pepper corns	~ 8 log cfu/g	Samples were subjected to steam treatment at 70 and 75°C for 5 min.	*E. faecium* was the most suitable surrogate to validate mild steaming processes.	Zhou et al., 2019

S. enterica and *L. monocytogenes*	*L. innocua* and *E. faecium*	Frozen blueberry	8.6 log cfu/g	Osmotically dehydrated and air-dried processes were carried out.	After osmotic dehydration of blueberries, only *E. faecium* was detected.	Bai et al., 2020
S. Senftenberg 775W, *S.* Enteritidis PT 30, & *S.* Thompson	*E. coli* P1, *E. faecium*	Basil leaves	~ 6 log cfu/g	Samples were dried at 60°C and 69°C for 90 min or at 100°C for 70 min using hot-air drying in the pilot scale dryer.	*E. coli* P1 was more suitable surrogate to validate hot-air drying processes compared to *E. faecium*	Zhou et al., 2020
S. Enteritidis PT 30	*E. faecium* NRRL B-2354	Pistachios	7.43 – 7.70 log cfu/g	Samples were heat treated in hot oil (121°C) or hot water (80°C) for 6 min. OR Samples were heat treated at 104.4 – 118.3°C with 30% or no humidity for 5 – 60 min.	*E. faecium* NRRL B-2354 is a good potential thermal-treatment surrogate for pistachios.	Moussavi et al., 2020
S. Typhimurium	*E. coli* AW1.7, *Pediococcus acidilactici*, *E. faecium* NRRL B-2354 and *S.carnosus*	Beef jerky	~ 8 log cfu/g	Samples were exposed to 60°C for 1, 4 or 15 min using a water bath.	*E. faecium* NRRL B-2354 and *S. carnosus* were validated as surrogate organisms for *Salmonella* after desiccation adaptation.	Schultze et al., 2020

Based on the studies summarized here, surrogate microorganisms can be obtained by genetic modification on the pathogens, isolating from original tested products, or adapting the surrogate already used to a new study matrix. Several studies used non-pathogenic *E. coli* or an avirulent strain of *S.* Typhimurium which contains no pathogenicity islands (Eblen et al., 2005, Jin et al., 2008, de Moraes et al., 2016) as surrogate microorganisms in lab-scale studies. This approach can surely mimic most characteristics of targeted pathogens except for pathogenicity. However, such selection is not recommended in an industry setting, because of the chance of reversion in pathogenicity or the occurrence of false-positive in the final products (Hu et al., 2017). Another method is to isolate surrogates from the original study matrix. Fudge et al. (2016) tested ten isolates from flour samples and suggested that one of these isolates, *P. dispersa* JFS, can work as a suitable surrogate for *Salmonella* in wheat flour during heat-treatment. Similarly, some commercial lactic acid bacterial cultures that have been used in making beef jerky or sausages can also be considered as a surrogate for *Salmonella* spp. in the respective products (Buege et al., 2006, Borowski et al., 2009). As listed in Table 1.4, several bacterial species have been recommended as *Salmonella* surrogates in different matrices. Among them, *Enterococcus faecium* NRRL B-2354 has been widely used as a surrogate for *S. enterica* for validating thermal processing of nuts and carbohydrate-protein meal (Jeong et al., 2011, Bianchini et al., 2014). Most importantly, the genetic safety of *E. faecium* NRRL B2354 as a surrogate has been thoroughly studied by Kopit et al. (2014) by sequencing, confirming that this strain lacks the genomic and phenotypic characteristics linked to nosocomial infections.

After selection, the surrogate microorganisms should be tested against targeted pathogens in a controlled lab study before conducting the validation study in a commercial setting or an open environment. *Salmonella* is considered as the most resistant non-spore forming bacterial pathogen to physical heat-treatment in various study matrices, such as in ground beef and pecans (Ma et al., 2007, Kharel et al., 2018). Heat resistance of bacterial cells can be affected by numerous factors, including the age of culture, the physiological status of culture (stress adapted or not) and inoculation methods (wet or dry) (O'Bryan et al., 2006). When evaluating the surrogate microorganism with a targeted pathogen in a validation study, all the aforementioned factors should be considered for both the surrogate and pathogenic microorganism (Ahmad et al., 2019, Schultze et al., 2020). According to these eligible studies, surrogate microorganisms should be selected with caution before testing against the target pathogen. Moreover, a relatively high inoculation level (6 to 8 log cfu/g or ml) was used in most validation studies to provide a sufficient log reduction for heat treatment.

Indicator microorganisms. The survival of pathogens in animal waste can also be predicted by monitoring indigenous bacterial species as indicator microorganisms. Studies suggested that indigenous enterococci or non-pathogenic *E. coli* could be a choice of indicator to validate the effectiveness of thermal processing for biosolid or animal waste (Côté et al., 2006, Graham et al., 2009, Chen et al., 2015). Côté et al. (2006) reported that 97.94-100% reduction of total coliforms and 99.67-100% reduction of indigenous *E. coli* resulted in undetectable levels of indigenous *Salmonella* during anaerobic digestion in swine slurries. The populations of indigenous enterococci and mesophiles ranged from 3.0-

7.5 log and 6.6-8.9 log cfu/g, respectively, in poultry litter during composting or stockpiling (Graham et al., 2009, Chen et al., 2015). These data suggested the raw poultry litter before physical heat treatment contained at least 3-log of naturally occurring enterococci, which can provide enough log-reduction for predicting the survival behaviors of *Salmonella* in poultry litter compost during thermal treatment.

As discussed above, except for the lab-scale validation studies, only pilot-scale models using pig slurry as matrix and indigenous microorganisms as indicators have been reported for animal waste treatment process validation (Cunault et al., 2011 & 2013). No validation study with representative test organisms (surrogate or indicator microorganisms) for heat-treated animal manure have been reported in the commercial industry settings in the U.S.

Competitive exclusion (CE) strategies to control pathogens

In addition to physical heat-treatment, other control strategies have also been developed to kill pathogens to ensure microbiological safety. Competitive exclusion (CE), also has been described as “bacterial antagonism” or “bacterial interference”, which is based on the involvement of non-pathogenic microorganisms to enhance the microbial competition in order to reduce pathogens in a certain environment (Nurmi et al., 1992). Traditionally, CE cultures isolated from animals have been added to the animal feed for synergistic interaction with the animal gut microbiota to reduce the pathogens in animal gut (La Ragione et al., 2003, Schneitz et al., 2005). When applied to the agriculture field

or food industry, CE cultures can be an excellent biological control tool that provides an environmentally friendly method to control foodborne or plant pathogens.

Isolation methods for CE microorganism. Culture-based methods are essential for isolating microorganisms, including CE microorganisms from numerous environments. The candidate CE strains can be isolated from environments where they have adapted. As shown in Table 1.5, CE microorganisms have been isolated from processing facilities or fecal samples that have no target pathogen contamination (Zhao et al., 2014, Danyluk et al., 2007) by directly culturing with nutrient media. After isolation, antagonistic activities of CE strains against pathogens can be quickly confirmed by spot-on-lawn for the isolates, patch plate or agar cylinder for cultured plates, and disc diffusion or agar-well diffusion methods for cell-free supernatant fluids. It is noteworthy that the spot-on-lawn is a preferred method if the CE microorganism is suspected to produce bacteriocin-like anti-bacterial compounds. This is because the bacteriocin-like compounds may be attached to the cell wall and thus the inhibition can only be effective when the cell is directly in contact with the pathogenic cell on agar surface (Ammor et al., 2006ab). However, the candidate CE species can be non-culturable or hard-to-culture microorganisms in natural environments, such as in soil or animal-waste. To the best of our knowledge, no published studies have been attempted to isolate such CE species from animal waste samples. Most CE microorganisms were isolated by plating target samples onto nutrient plates followed by confirmation of antagonistic activities against pathogens (Table 1.5). Therefore, special stimuli such as adding specific growth nutrients or growth promoting factors, modification of isolation agar preparation, using extended incubation time or reduced-strength nutrient

cultures, should be taken into account for CE isolation or resuscitation of viable but non-culturable or hard-to-culture microorganisms from environmental samples (Pulschen et al., 2017, Zhao et al., 2017).

Table 1.5 Methods for isolating CE microorganisms to control major foodborne pathogens since 2000.

Isolation matrix	Isolation or screening methods	Comments	Reference
Biofilm samples collected from floor drains at food processing plants	Spot-on-lawn: Samples were plated onto nutrient agar, followed by spot-on-lawn inoculation using double-layer assay.	Bacterial isolates were identified as lactic acid bacteria.	Zhao et al., 2004
Dry sausages processing facility	Agar well diffusion and overlay agar assay: The bacterial culture or cell-free culture supernatant was inoculated into agar well.	The production of bacteriocins only on agar plated in overlay assays, not in cell-free culture supernatant.	Ammor et al., 2006
Fresh peeled baby carrots	Spot-on-lawn and growth on pepper disk.	*Pseudomonas fluorescens* 2-79 or *Bacillus*YD1 at 5 to 6 log CFU/g as used in this study can provide 3.8-4.0 log reduction of foodborne pathogens.	Liao et al., 2009
Raw milk sample and feces sample	Spot-on-lawn using double-layer assay.	Lactic acid bacteria isolated from raw milk had a low antagonistic activity against *E. coli*. 25 CE strains were isolated from feces samples.	Danyluk et al., 2007; Tamanini et al., 2012
Fern plant	Patch plate method: Bacterial isolates were patched inoculated onto plates.	Endophytic bacteria [a] can produce antibiotic substances that were capable of controlling *L. monocytogenes*, *B. ce-reus*, *S. aureus*, *E. coli*, and *S. Typhimurium.*	Das et al., 2017
Soil samples	Agar cylinder diffusion assay: agar cylinder was cut and removed from the agar plates inoculated with diluted soil sample after 2-days growth.	The purified isolates of actinomycetes belonged to *Streptomyces* spp. But some inhibition was not so clear due to the cell morphology.	Benreguieg et al., 2017
Dairy products	Involved enrichment step: Samples were enriched first in MRS broth, then spread plated onto MRS agar, followed by confirmation using spot-on-lawn.	The enrichment step can promote the isolation of *Lactobacillus* from dairy product.	Karami et al., 2017
Kefir and kefir grains	Triple-agar-layer.	The second layer of agar supplemented with Natamycin can promote the fungal growth.	Powell et al., 2007

[a] Endophytic bacteria: *Bacillus* sp. *cryopeg*, *Paenibacillus Staphylococcus warneri*, and *Bacillus psychrodurans*

Mechanisms of competitive exclusion (CE) strategies. The mechanisms of CE microorganisms inhibiting human pathogens in the natural environment include the production of antibacterial substances and competition for limiting nutrient sources or attachment sites on the favorable surface. For some situations, a synergistic interaction of two or more of these activities can occur. When the antimicrobial compounds are produced by CE microorganisms, the antibiotics should be produced at a sufficient level to effectively suppress the growth of pathogens. According to the literature, the antibiotic production is regulated by the quorum-sensing mechanism (Moslehi-Jenabian et al., 2011). When different species of bacteria co-exist in one environment, the higher growth rate and the ability of up-taking the limited amount of essential nutrients from the growth environment are key factors for establishing the dominance by the CE strains (Hibbing et al., 2010). For example, the siderophore production for acquiring iron and the competitive uptake of glucose have been proved as key mechanisms of inhibiting the growth of fish pathogen (*Aeromonas hydrophila*) by *Bacillus cereus* (Lalloo et al., 2010).

The competition for the attached site occurs between CE microorganisms and pathogens can be either the co-attachment on the same surface or the displacement of the existing pathogen colonization by CE. The capability of the selected *Lactobacillus* strain to displace pathogen colonization on the mucosal surface was confirmed by Gueimonde et al. (2006). As highly motile cells can access more nutrients, the motility can contribute to the dispersal and affect the competitive activity of bacteria (Hibbing et al., 2010). Therefore, the use of high-motile microorganisms as CE candidates should be taken into further consideration.

Application of CE strategies to control plant pathogens in the agricultural field as biological control agents. CE microorganism can be applied to suppress plant/soilborne pathogens (Köhl et al., 2019), which have been known as biological control agents. In fact, although crop losses caused by plant pathogens alone are hard to estimate, plant disease caused by plant pathogens is a major contributor to the crop yield loss (ca. $60 billion worldwide) (Loebenstein et al., 2009), posing an economic benefit of using biological control to defeat plant disease.

Plant pathogens can induce plant diseases, such as *Rhizoctonia solani* (the cause of damping-off and loss of crop yield) (Qian et al., 2009, Liu et al., 2018c), *Fusarium oxysporum* (the cause of vascular wilts) (Qiu et al., 2012, Xue et al., 2015), and *Erwinia amylovora* (the cause of fire blight disease of pear) (Sharifazizi et al., 2017). Numerous beneficial microorganisms, such as *Bacillus subtilis* or *Bacillus* spp., *Lactobacillus plantarum*, *Pseudomonas* spp., *Pantoea agglomerans*, *Rahnella aquatilis*, *Trichoderma asperellum*, or other yeasts have been used as biocontrol agents against plant pathogens (Zeller et al., 2006, Fira et al., 2018, Postma et al., 2019, Al-Ghafri et al., 2020). Some of these organisms have become commercially available for treating plant diseases (Pastrana et al., 2016, Smolińska et al., 2018). There are growing interests and opportunities in using microbial biological control agents against plant diseases. Note that the biological control agents should be added accordingly with the pathogen development such asin the early stages in order to reach a stable beneficial microbial community prior to the invasion of pathogens (Postma et al., 2008).

Applications of CE strategies to control foodborne pathogens. In addition to plant pathogens, research on CE has been traditionally focused on controlling the colonization of *Salmonella* in gastrointestinal tracts of chickens (Vandeplas et al., 2008). When CE cultures are used in animal feed, CE cultures can promote the heathy host immune system. These kinds of microorganisms can work as probiotics for farm animals (Callaway et al., 2008). Promising results have been reported for lactic acid bacteria (LAB) culture controlling of *E. coli*, *Yersinia pseudotuberculosis*, and *S. enterica* in chicken, cattle, and pig (Anderson et al., 1999, Brashears et al., 2003). Essentially, the most common microbial genera used as probiotics in different studies are *Enterococcus, Bifidobacterium,* nonpathogenic *Escherichia, Lactobacillus* and *Saccharomyces* (Wan et al., 2019). These probiotics have been used as an alternative to antimicrobials in animal feeds, and conferred benefits to the host gut. Specifically, the use of these CE cultures in animal feed not only serves as a competitive function against pathogens but can also enhance the yield of vitamins and antioxidants produced in the host animals (Amaretti et al., 2013).

Published literature reviews have focused primarily on using CE as probiotics for farm animals (Callaway et al., 2008, Wan et al., 2019), and the potential use of CE cultures in the food industry in recent years has not been reviewed in detail. Therefore, a systematic literature search was performed to identify competitive exclusion strategies used to control major foodborne pathogens from farm to food processing plants. As stated in previous sections, EBSCO (Academic Search Complete) and Web of Science, were searched for peer-reviewed articles published from 2000 to 2020. The search strings and study selection

procedure are listed in Table A3 and Figure A3, respectively (Appendix A). After selection and quality assessment, 32 eligible studies were summarized in Table 1.6.

Table 1.6 Summary of application of CE strategies to control major foodborne pathogens since 2000 as indicating by major CE species, inoculation used, target pathogens and study matrix.

CE species	CE Inoculation level	Targeting foodborne pathogens/level	Study matrix and test methods	Reference
Lactic acid bacteria including *Lactobacillus* spp., *Enterococcus durans*	7 log cfu/g	*E. coli* O157: H7 and *L. monocytogenes* / 5.5 log cfu /g	Cut cabbage	Harp et al, 2003
	5 log cfu/ml	*L. monocytogenes* / 3 log cfu/ml	Co-culture in TSB-YE and biofilms formation on stainless steel coupons	Zhao et al., 2004
	9 log cfu/ml	*L. monocytogenes* / 3.6 – 7.5 log cfu/ 100 cm^2	Floor drains of a poultry processing plant	Zhao et al., 2006
	7 log cfu/ml	*L. innocua*, *S. aureus* or *Hafnia alvei* / 5 log cfu/ml	Biofilm growth model	Ammor et al., 2006
	3-4 log cfu/g	*L. monocytogenes* / 3-4 log cfu/g	Co-culture in sliced sausage with different packaging types	Kaban et al., 2010
	N.A.	*L. monocytogenes* and *E. coli* / 8 log cfu/ml	Raw milk sample with spot-on-lawn	Tamanini et al., 2012
	8 log cfu/ml	*Salmonella* /8 log cfu/ml	Co-culture in mixed culture	Szala et al., 2012
	5 log cfu/ml	*L. monocytogenes* / 5.5 log cfu/ml	Cheese and biofilm	Ben et al., 2013
	6 log cfu/ml	*L. monocytogenes* / 3 log cfu/g	Co-culture in cheese	Samelis et al., 2017
	Biofilm formed by CE	*L. monocytogenes* / 8.01 log cfu/ml biofilm	Biofilm formed by CE	Turhan et al., 2017

	with 9.46 and 9.66 log cfu/ml CE load			
	N.A.	*S. aureus, B. subtilis,* and *P. aeruginosa* /overnight culture	Spot-on-lawn	Karami et al., 2017
	9 log cfu/ml	*L. monocytogenes* / at 4°C: 7.1-7.7 log cfu/cm^2 at 8°C: 7.5-8.3 log cfu/cm^2	Biofilms on coupons composed of different materials (stainless steel, plastic, rubber, glass, and silicone)	Zhao et al., 2013
	2% LAB culture	*L. monocytogenes* / 4-6 log cfu/ml	Co-culture in cheese	Kondrotiene et al., 2018
	8 log cfu/ml	*L. monocytogenes* / 4-5 log cfu/ml	Biofilm on stainless steel	Dygico et al., 2019
	7 log cfu/ml	*E. coli* O157: H7, *B. cereus*, and *S. aureus* / 6 log cfu/ml	Agar well diffusion	Hafez et al., 2019
	7 log cfu/ml	*Salmonella* /7 log cfu/ml	Co-culture in mixed culture	Shi et al., 2019
	Ca. 6 log cfu/ml	*L. monocytogenes*, *E. faecalis*, and *S. aureus* / 4-5 cfu/ml	Fresh pork sausage	Gelinski et al., 2019
Pseudomonas spp.	5 log cfu/ml	*Salmonella* / 3 log cfu/ml	Co-culture in TSB and alfalfa seed soak water	Fett et al., 2006
	Ca. 7 log cfu/ml	*L. monocytogenes* and *Salmonella* /5 log cfu/ml	Fresh-cut pear	Iglesias et al., 2018
	7 log cfu/ml	*L. monocytogenes*/5 log cfu/ml	Spot-on-lawn, and co-culture in melon plugs, and melon juice	Collazo et al. 2017
Pseudomonas fluorescens AG3A (Pf AG3A*)* and Pf 2-79, and *Bacillus* YD1	5 -8 log cfu/ml	*E. coli* O157: H7, *L. monocytogenes*, *Salmonella*, and *Yersinia enterocolitica* / 5 log cfu/ml	Co-culture in TSB	Liao et al., 2009

Streptomyces spororaveus, Bacillus safensis, and Pseudomonas azotoformans	Biofilm formed by CE with 7.9-8.5 log cfu/coupon CE load	*S. aureus* / 4.2 log cfu/ coupon	Biofilm formed by CE on stainless steel	Son et al., 2016
Pseudomonas extremorientalis, Paenibacillus peoriae, and *Streptomyces cirratus*	8.6, 8.8, and 6.4 log cfu/coupon	*Salmonella* / 4.1 log cfu/coupon	Biofilm formation on stainless steel surface	Kim et al., 2018
Bacillus	Cell-free supernatants	*B. cereus, E. coli* O157: H7, *L. monocytogenes*, *Salmonella*, *S. aureus*, *P. aeruginosa*	Disc diffusion assay	Avci et al., 2016
Endophytic bacteria: *Bacillus* sp. *cryopeg*, *Paenibacillus Staphylococcus warneri*, and *Bacillus psychrodurans*	N.A.	*B. cereus, E. coli* O157: H7, *L. monocytogenes*, *Salmonella*, *S. aureus*	Spot-on-lawn	Das et al., 2017
Paenibacillus polymyxa	6 log cfu/ml	*E. coli* O157: H7 / 2, 3, 4, or 5 log cfu/ml	Biofilm formed by CE	Kim et al., 2013
Leuconostoc	5-9 log cfu/g	*L. monocytogenes* / 3-4 log cfu/g	Co-culture on wounds of fruit and vegetable	Trias et al., 2008
Streptomyces	2-day old culture	*L. monocytogenes* / 24 h – culture	Agar cylinder diffusion assay	Benreguieg et al., 2017
Phyllosphere-associated lactic acid bacteria	4 log cfu/ 5 cm^2	*Salmonella* / 3 log cfu/ 5 cm^2	Co-culture on the surfaces of cantaloupes	McGarvey et al., 2019

Erwinia persicina	5-8 log cfu/ml	*Salmonella* / 3 log cfu/ml	Spot-on-lawn and co-culture in alfalfa seed soak water	Kim et al., 2020
Comme Competitive Inhibition Bacteria of Bovine Origin against *Salmonella* Serovarsrcial probiotic supplement *Lactobacillus rhamnosus* GG (LGG) (Culturelle®)	9 log cfu/g for coculture	*Salmonella,* and *L. monocytogenes* / 3-4 log cfu/g for co-culture	Spot-on-lawn and co-culture in cook-chill cream of potato soup	Muñoz et al., 2019
Commercially protective bacterial cultures [a]	9 log cfu/ml	*L. monocytogenes*, *Salmonella*, and STEC / 7 log cfu/ml	Spot-on-lawn	Gensler et al., 2020

[a] Commercially produced PCs used were *Lactococcus lactis* subsp. *lactis* BS-10 (LLN), *Pediococcus acidilactici* B-LC-20 (PA), *Lactobacillus curvatus* B-LC-48 (LC) (Chr. Hansen Inc., Milwaukee, WI), *Lactobacillus plantarum* (LPP) Holdbac Listeria (DuPont Danisco USA Inc., New Century, KS), *Lactobacillus rhamnosus* Lyofast LRB (LR), *Lactobacillus plantarum* Lyofast LPAL (LP), *Carnobacterium* spp. Lyofast CNBAL (CS) (Sacco Srl, Amerilac, Miami, FL), LALCULT Protect *Hafnia alvei* B16 (HA), LALCULT Protect *Staphylococcus xylosus* XF01 (SX) (Lallemand Specialty Cultures, Blagnac, France), and *Enterococcus faecium* SF68 (EF) (NCIMB 10415, Cerbios-Pharma SA, Barbengo, Switzerland)

Table 1.6 lists the CE cultures that have been widely used in controlling foodborne pathogens, including lactic acid bacteria, *Enterococcus*, *Pseudomonas*, *Paenibacillus*, *Streptomyces*, *Bacillus*, and some commercially protected bacterial cultures. Major foodborne pathogens have been targeted, including *L. monocytogenes*, Shiga toxin producing *E. coli* (STEC), *Salmonella*, *B. cereus*, and *S. aureus* at a level of 3 to 8 log cfu/g or ml. The application of defined or undefined CE cultures at the levels of 3 to 9 log cfu/g or ml has been used to decrease pathogen populations and avoid cross-contamination in various study matrix, like in co-culture, biofilm, fresh produce, packaged food, dairy products, and food processing facilities. Different methods for testing the antagonistic activities have been used. The inhibition effects from CE on foodborne pathogens as pathogen reductions (no reduction to > 7 log-reduction) or zone of inhibitions (2 mm to 3 cm) differed among studies. In general, reduction of pathogen increased with the increasing CE concentration due to more antimicrobial compounds produced or the effect from population competition (Liao et al., 2009, Kim et al., 2020).

The CE isolates can be applied to control foodborne pathogens in different environments. CE cultures can inhibit the growth potential of pathogenic bacteria, when CE cultures were directly applied to decompose the pathogenic biofilms. Likewise, studies have reported that the growth potentials of *L. monocytogenes*, *Salmonella*, *L. innocua*, *S. aureus*, and *H. alvei* can be reduced by CE culture treatment by 2- to 6-log (Zhao et al., 2004, 2006 & 2013, Ammor et al. 2006). The biofilm formed by CE strains also can emerge as a protective barrier to reduce the pathogen contamination. For example, populations of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, and *Salmonella*, decreased by 0.4 – 2 log

cfu/coupon after inoculation on the stainless-steel coupon contains preformed biofilm by CE (Kim et al., 2013, Son et al., 2016, Kim et al., 2018). When CE treatment was applied to fresh produce and packaged foods, the efficacy of CE treatment was affected by the vegetable type and packaging materials. For example, Harp et al. (2003) found there was no noticeable antagonistic action against *E. coli* O157: H7 and *L. monocytogenes* induced by *Lactobacillus,* probably due to the catalase activity in the cut cabbage. In contrast, the use of *L. sakei* with modified atmosphere packaged sausage has a synergistic inhibitory effect on controlling the post-processing contamination in cooked meat products with *L. monocytogenes* (Kaban et al., 2010). Clearly, the findings from published studies have provided novel insights into practical use of CE microorganisms to control foodborne pathogens in different environmental niches.

Suppression of pathogens by CE isolated from compost. As a nutrient-rich ecosystem, the rhizosphere is known to contain highly competitive activities among microbial communities. Studies also revealed that the application of organic compost as fertilizer to soil can suppress soilborne pathogens by regulating microbial community in rhizosphere (Nega 2014). Several beneficial microorganisms with antagonistic activities against soilborne pathogens were identified from compost (Ren et al., 2012, Mulero-Aparicio et al., 2020, Al-Ghafri et al., 2020). For example, in a recent study, several bacterial strains isolated by Al-Ghafri et al. (2020) from compost were screened for their inhibition ability against plant pathogens. As a result, the antagonistic activity of *Pseudomonas aeruginosa* against *Pythium aphanidermatum* and *Fusarium solani* was confirmed by the observation of the morphological change of the pathogen under electron

microscope. Furthermore, some beneficial microorganisms have been added to thermophilic composting stage to increase the soilborne pathogen suppression in the compost (Mulero-Aparicio et al., 2020, Al-Ghafri et al., 2020). Nonetheless, there are very limited studies documenting the isolation and use of CE as a biological control agent to eliminate human pathogens in animal waste or other soil amendments. Only Puri et al. (2010) performed a lab-scale study of investigating the survival of *E. coli* O157: H7 in compost slurry and reported the presence of cycloheximide-sensitive eukaryotic species can reduce the growth of spiked *E. coli* O157: H7 by ca. 4-log in the compost.

Although competitive exclusion of pathogens in different environments has been reported, studies between foodborne pathogens and CE strains have yielded no effective CE microorganisms from animal waste or animal waste-based compost to inhibit foodborne pathogens such as *L. monocytogenes*. Many studies have concluded that microbial diversity is a key barrier against pathogen contamination in a various matrix, such as rhizosphere, mouse gut, and soil (De Brito et al., 1995, Vivant et al., 2013, Gurtler et al., 2017, Ji et al. 2017). Hence, it is important to further study the microbial community in animal waste or animal waste-based compost to determine if beneficial CE cultures can be isolated

Microbial community in animal waste

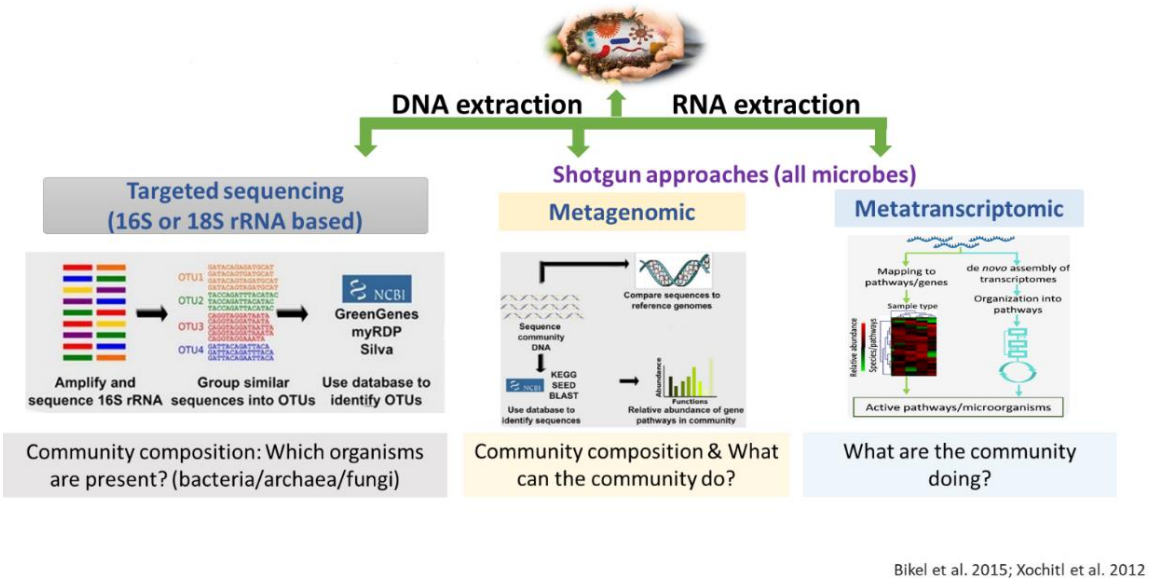
Animal waste-based compost is rich in numbers and variety of microorganisms, especially those beneficial microorganisms, which may mediate suppression of foodborne pathogens even in the finished compost. Microbial communities in animal waste

ecosystems can influence a range of complex interactions among microorganisms, including interactions with other microbes in the shared environment and competitive exclusion among others. Like in most environments, such as soil or animal waste, more than 90% of microorganisms cannot be cultured (Cytryn et al., 2013). Therefore, it is essential to shift the emphasis from traditional culturing method to the culture-independent techniques (Riesenfeld et al., 2004).

Traditional approaches on characterizing a microbial community. The compost microbiome plays important roles in cthe ompost ecosystem, ranging from regulating the composting process, providing fertility to crops, and serving as a source of beneficial bacteria (Autunes et al., 2016, Agrillo et al., 2019). Therefore, it is essential to characterize the microbial communities found in compost. A number of techniques have been involved in studying the microbiota, which can be divided into the following groups: 1) community-level physiological profiling or metabolic potential analysis (e.g. Ecoplates, MicroPlates from Biolog) (Hueso et al., 2012, Liu et al., 2014); 2) DNA-based fingerprinting methods including cloning and sequencing, restriction fragment length polymorphism, automated ribosomal intergenic spacer analysis, terminal restriction fragment length polymorphism, and denaturing/temperature gradient gel electrophoresis, (Ercolini et al., 2004, Nocker et al., 2007, Xiao et al., 2011). Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting was widely used to analyze the microbial community in compost. However, the potential bias includes PCR product purification and the final resolution of the gel image. In recent decades, the in-

depth screening of microbial community in environmental samples was achieved by next-generation sequencing (NGS).

Next-generation sequencing approach. The sequencing methods frequently used in microbial community analysis include the targeted 18S/16S rRNA gene sequencing (rDNA sequencing), shotgun metagenomics sequencing, microbial transcriptome analysis, and whole-genome microbial sequencing. Following sequencing, bioinformatics and multivariate statistical analyses can provide a detailed assessment of microbial composition and functions from the complex compost-microbial communities (Paliy et al., 2016). General workflow and differences among these sequencing approaches are shown in Figure 1.3 (Morgan et al., 2012, Bikel et al., 2015).



Bikel et al. 2015; Xochitl et al. 2012

Figure 1.2 Workflow for targeted, metagenomic, and metatranscriptomic sequencing approaches (Bikel et al., 2015, Aguilar et al., 2016)

Targeted sequencing (amplicon sequencing). The16S/18S rRNA gene sequencing is commonly used to identify and compare microbial community within a sample (Klindworth et al., 2013). Total genomic DNA extracted from samples is subjected to phylogenetic marker analysis based on the sequencing of 16S rRNA gene for bacteria and 18S rRNA gene for eukaryotes to profile the microbial communities of study samples (Klindworth et al., 2013). In general, there are three reasons to use 16S rRNA gene sequencing to study bacterial community, including: 1) the length of the 16S rRNA gene (~1,500 bp) is capable of providing enough genetic information; 2) the wide presence of this genetic marker in almost all bacteria; 3) the stable function of 16S rRNA gene over time. To date, there are 3,340 bacterial and archaea bacterial genera recognized and published (ICNP, 2020).

Sequences generated from the targeted sequencing are clustered into Operational Taxonomic Units (OTU), followed by comparing against databases to identify the microbial members present in the ecosystem. Several bioinformatics tools can be used for 16S/18S rRNA gene sequencing data analysis, including Mothur, Quantitative Insights Into Microbial Ecology (QIIME), and QIIME2 (Caporaso et al., 2010, Schloss et al., 2009, Bolyen et al., 2019). López-García et al. (2018) compared the outputs from Mothur and QIIME using data generated from rumen content of dairy cows, and found that except for some uncommon microorganisms, both Mothur and QIIME can reveal comparable richness and diversity for microbial ecology datasets they have used. However, this conclusion should be interpreted with caution, because workflows including DNA extraction from the study matrix, library construction, and sequencing platform, can affect

the sequencing data quality, thus bioinformatics pipeline should be optimized according to a specific project (Allali et al., 2017). Community data, including taxonomic distribution, alpha diversity, and beta diversity, are used to show the characteristics of microbial compositions, species richness/diversity detected in a microbial ecosystem, and differences among microbial communities from different environments, respectively (Hugerth et al., 2017).

The use of 16S/18S rRNA gene sequencing analysis has been widely used to characterize the microbial community structure in animal waste (Neher et al., 2013, Pandey et al., 2018, Ma et al., 2018, Zhong et al., 2018). As reported by Neher et al. (2013), both bacterial and fungal communities responded to the change in compost recipe and methods. Abundances of *Firmicutes*, *Actinobacteria*, *Gemmatimonadetes*, and *Chloroflexi* were found to be significantly different between hay and hardwood recipes for compost. Some fungi associated with tree bark, like *Sordariomycetes* and *Agaricomycetes*, were predominant in the hardwood recipe. In the same study, microbial communities observed in vermicompost differed from those in windrow compost, which can be explained by differences in composting temperature. Using 16S rRNA gene sequencing, the predominant bacterial members in animal waste-based compost have been identified, such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi*, as these phyla contain species that are actively involving in decomposition of lignocellulose and complex organic compounds in animal manure (Zhong et al., 2018, Pandey et al., 2018).

Shotgun metagenomics sequencing. To target all the microbes in the study matrix, shotgun metagenomics/metatranscriptomic sequencing should be used to sequence the

entire DNA/RNA from a sample (Cao et al., 2017). Unlike targeted sequencing, metagenomic data not only provides the taxonomic information of bacteria/fungi but can also identify the relative abundance of all organisms and provide comprehensive information on microbial community structure and functional potential (Shah et al., 2011). Meneghine et al. (2017) used the metagenomic approach to study microbial interactions, in terms of taxonomic and functional profiles of microbiomes in irrigation water, organic fertilizer and soil. Results from that study indicated that nitrogen fixation plays a crucial role in the reported ecosystem. However, one of the limitations of genomic DNA sequencing analysis is the inability to differentiate live (dormant cells as well as growing or non-growing metabolically active cells) and dead cells.

To avoid the extraction of DNA from dead microorganisms, a viability assay with propidium monoazide (PMA) has been widely used for pretreatment of complex samples prior to microbial total DNA extraction (Li et al., 2017). The pre-treatment step using PMA inhibits the PCR-amplification DNA from dead cells and has been used in combination with end-point PCR, gene enumeration, and microbial community analysis (Carini et al., 2016, Heise et al., 2016). Despite promising results, several factors should be considered for a successful experiment design, including concentration of PMA dye, incubation time that allows the chemical to enter the cells and contact its DNA, the light source used to make reaction happens, the proper preparation of samples, and how to completely remove the PMA from treated samples (Fittipaldi et al., 2012). To resolve these challenges, treatment procedures can be optimized using qPCR prior to sequencing analysis.

Shotgun metatranscriptomic sequencing. Alternatively, by investigating genes that are expressed by the entire microbial community, metatranscriptomic sequencing can provide the active functional profile in an ecology system (Gilbert et al., 2011). In recent years, metatranscriptomic sequencing is a valuable approach to learn the active functional metabolic profiles during composting process in compost ecosystems. Through metatranscriptomic study on thermophilic composting, Autunes et al. (2016) reported that lignocellulose degradation in a thermophilic composting process is exclusively the result of bacterial enzymatic activities, and proposed microbial members, like *Actinomycetales*, *Bacillales*, *Clostridiales*, and *Enterobacteriales* are primarily responsible for lignocellulosic biomass degradation. In another study using metatranscriptomic sequencing, Wang et al. (2017) identified a core resistome during composting process. However, it is not surprising that the difficulty in obtaining enough high-quality mRNA with complex environmental samples, such as animal waste or animal waste-based compost. As a result, studies on the microbial communities from such a matrix have often been used DNA rather than RNA sequencing (Moran et al., 2009).

Available workflows for metagenomic/metatranscriptomic pipelines. For the downstream analyses of metagenomic data, the richness and diversity measurements of taxa information were like those of targeted sequencing analysis. The most essential data obtained from metagenomic sequencing is the functional potentials mapped with the sequencing reads. Several useful annotation resources include gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups (COG) are available for functional annotation. These sources are generally incorporated

with the analysis tools. For metatranscriptomic sequencing, reads are mapped into databases using alignment tools before annotation using the aforementioned resources. Currently, metagenomic/metatranscriptomic analysis pipelines are available as web-based user interfaces for performing these steps (Aguiar-Pulido et al., 2016, Shakya et al., 2019).

Because of the broad information including both microbiome abundance and gene expression profiles, both metagenomics and metatranscriptomic sequencing techniques can generate many data reads, and any form of sequencing data analysis is still of great focus for future efforts. Nonetheless, the pre-quality control of metagenome data should be performed prior to the bioinformatics analysis.

Application of NGS on the interaction between human pathogens and background microorganisms. As alluded to above, the involvement of technologies such as high-throughput sequencing may allow us to understand microbial interactions at the community level in greater depth. In food processing facilities, the microbiome of *Listeria*-colonized and *Listeria*-free drains and apple washing conveyor belt were characterized as different, indicating the occurrence of *Listeria* was closely associated with the background microbiota in these built environments (Fox et al., 2014, Tan et al., 2019). In the animal intestinal ecosystem, the host-pathogen interactions have been extensively reported (De Jong et al., 2012, Ji et al., 2017). However, there are few published studies focused on how the indigenous microflora respond to the invasive pathogenic bacteria in soil (Vivant et al., 2013, Falardeau et al., 2018, Schierstaedt et al., 2020). By building up the constructed microcosms using serially diluted soil samples (10^8 – 10^2 cfu/ml), Vivant et al. (2013) found that there was a negative correlation between the level of diversity and the survival

rate of spiked *L. monocytogenes*. Similarly, Schierstaedt et al. (2020) demonstrated the abundance of inoculated *Salmonella* decreased in soil with higher diverse indigenous microbial communities. Although these two studies have been performed using a dilution-to extinction approach with few samples, high species diversity of animal waste or animal waste-based compost can be an effective biological barrier that eliminates the invading pathogens.

In short, despite a few metagenomics/metatranscriptomic sequencing studies of the thermophilic composting process (Antunes et al., 2016), no studies have been carried out on the functional metatranscriptomics of human pathogen interactions with indigenous microorganisms in animal waste-based biological soil amendments, albeit there are no published studies about comparing microbial communities in commercial animal waste-based composts. In considering of compost as a rich source of microorganisms with a diversity of microbial species, the large NGS sequencing datasets generated from animal waste-based composts can surely be a great source to identify unique microbial members for controlling pathogens in various environments.

Summary

Animal waste or animal waste-based compost, commonly used as organic fertilizer, may contain human pathogens such as *Salmonella* and *L. monocytogenes*. *Salmonella* is more frequently isolated from poultry litter or poultry litter compost. Physical heat treatments are commonly used to inactivate *Salmonella* in poultry litter with or without composting process, but the validation study or guidelines are still needed for the litter

processing industry to ensure microbial safety of their products. On the other hand, due to the ubiquitous nature of *L. monocytogenes*, it is essential to understand the ecology of this pathogen where it inhabits and then develops strategies to reduce *Listeria* contamination. We hypothesized that the compost-adapted competitive exclusion (CE) microorganisms against *L. monocytogenes* exist in animal waste-based compost. In combination with the culturing method, the use of NGS sequencing approaches is expected to guide us for discovering those highly adapted CE microorganisms in composts for controlling *L. monocytogenes* in produce growing and processing environments.

Therefore, the objectives of this work were to:

1). Test a nonpathogenic surrogate microorganism for validating desiccation-adapted *Salmonella* inactivation in physically heat-treated broiler litter.

2). Validate the physical heat treatment of poultry litter composts using surrogate and indicator microorganisms for *Salmonella* in industrial settings.

3). Use sequencing approaches to understand the microbial community profile and functions in compost in the presence and absence of *L. monocytogenes*.

4). Isolate and identify the competitive exclusion microorganisms against *L. monocytogenes* in biological soil amendments.

REFERENCES

1. Agrillo B, Mirino S, Tatè R, Gratino L, Gogliettino M, Cocca E, Tabli N, Nabti E, Palmieri G. 2019. An alternative biocontrol agent of soil-borne phytopathogens:

A new antifungal compound produced by a plant growth promoting bacterium isolated from North Algeria. Microbiol Res 221:60-69.

2. Aguiar-Pulido V, Huang W, Suarez-Ulloa V, Cickovski T, Mathee K, Narasimhan G. 2016. Metagenomics, metatranscriptomics, and metabolomics approaches for microbiome analysis: supplementary issue: bioinformatics methods and applications for big metagenomics data. Evol. Bioinform. 12:EBO. S36436.
3. Ahmad NH, Oztabak C, Marks BP, Ryser ET. 2019. Effect of Talc as a Dry-Inoculation carrier on thermal resistance of *Enterococcus faecium* NRRL B-2354 in almond meal. J Food Prot 82:1110-1115.
4. Alali WQ, Thakur S, Berghaus RD, Martin MP, Gebreyes WA. 2010. Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms. Foodborne Pathog Dis 7:1363-1371. doi:10.1089/fpd.2010.0566 [doi].
5. Albino LA, Rostagno MH, Hungaro HM, Mendonca RC. 2014. Isolation, characterization, and application of bacteriophages for *Salmonella* spp. biocontrol in pigs. Foodborne Pathog Dis 11:602-609. doi:10.1089/fpd.2013.1600 [doi].
6. Al-Ghafri HM, Velazhahan R, Shahid MS, Al-Sadi AM. 2020. Antagonistic activity of *Pseudomonas aeruginosa* from compost against *Pythium aphanidermatum* and *Fusarium solani*. Biocontrol Sci Technol 1-17.
7. Allali I, Arnold JW, Roach J, Cadenas MB, Butz N, Hassan HM, Koci M, Ballou A, Mendoza M, Ali R. 2017. A comparison of sequencing platforms and

bioinformatics pipelines for compositional analysis of the gut microbiome. BMC Microbiol.17:194.

8. Amaretti A, Di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A. 2013. Antioxidant properties of potentially probiotic bacteria: in *vitro* and in *vivo* activities. Appl Microbiol Biotechnol 97:809-817.
9. Ammor S, Tauveron G, Dufour E, Chevallier I. 2006a. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1—Screening and characterization of the antibacterial compounds. Food Control 17:454-461.
10. Ammor S, Tauveron G, Dufour E, Chevallier I. 2006b. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 2—Behaviour of pathogenic and spoilage bacteria in dual species biofilms including a bacteriocin-like-producing lactic acid bacteria. Food Control 17: 462-468.
11. Anderson RC, Stanker LH, Young CR, Buckley SA, Genovese KJ, Harvey RB, DeLoach JR, Keith NK, Nisbet DJ. 1999. Effect of competitive exclusion treatment on colonization of early-weaned pigs by *Salmonella* serovar Choleraesuis. J Swine Health Prod 7:155-160.
12. Antunes LP, Martins LF, Pereira RV, Thomas AM, Barbosa D, Lemos LN, Silva GMM, Moura LMS, Epamino GWC, Digiampietri LA. 2016. Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. Sci. Rep. 6:38915.

13. Arrus KM, Holley RA, Ominski KH, Tenuta M, Blank G. 2006. Influence of temperature on *Salmonella* survival in hog manure slurry and seasonal temperature profiles in farm manure storage reservoirs. Livest. Sci. 102:226-236.

14. Avcı A, Üzmez S, Alkan FB, Bagana İ, Nurçeli E, Çiftçi E. 2016. Antimicrobial activity spectrums of some *Bacillus* strains from various sources. GIDA/The Journal of Food 41:.

15. Avery LM, Williams AP, Killham K, Jones DL. 2009. Heat and lime-treatment as effective control methods for *Escherichia coli* O157: H7 in organic wastes. Bioresour Technol 100:2692-2698.

16. Bai X, Campagnoli M, Butot S, Putallaz T, Michot L, Zuber S. 2020. Inactivation by osmotic dehydration and air drying of *Salmonella*, Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, hepatitis A virus and selected surrogates on blueberries. Int J Food Microbiol 320:108522.

17. Bang J, Choi M, Jeong H, Lee S, Kim Y, Ryu J, Kim H. 2017. Heat tolerances of *Salmonella*, *Cronobacter sakazakii*, and *Pediococcus acidilactici* inoculated into galactooligosaccharide. J Food Prot 80:1123-1127.

18. Ben Slama R, Kouidhi B, Zmantar T, Chaieb K, Bakhrouf A. 2013. Anti-listerial and Anti-biofilm activities of potential probiotic *Lactobacillus* strains isolated from Tunisian traditional fermented food. J Food Saf 33:8-16.

19. Benreguieg M, Hachem K, Adli D, Kaid M. 2017. Production and partial characterization of antimicrobial agent from Algerian Arid Soil *Streptomyces. Transylvanian* Review 1:.

20. Bianchini A, Stratton J, Weier S, Hartter T, Plattner B, Rokey G, Hertzel G, Gompa L, Martinez B, Eskridge KM. 2014. Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. J Food Prot 77:75-82.

21. Bikel S, Valdez-Lara A, Cornejo-Granados F, Rico K, Canizales-Quinteros S, Soberón X, Del Pozo-Yauner L, Ochoa-Leyva A. 2015. Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. Comput. Struct. Biotechnol. 13:390-401.

22. BioCycle. Available on https://www.biocycle.net/2017/10/04/state-organics-recycling-u-s/.

23. Biswas S, Niu M, Pandey P, Appuhamy J, Leytem A, Kebreab E, Dungan R. 2018. Effect of dairy manure storage conditions on the survival of *Escherichia coli* O157: H7 and *Listeria*. J Environ Qual 47:185-189.

24. Biswas S, Pandey PK, Farver TB. 2016. Assessing the impacts of temperature and storage on *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes* decay in dairy manure. Bioprocess Biosyst Eng. 39:901-913.

25. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852-857.

26. Borowski AG, Ingham SC, Ingham BH. 2009. Validation of ground-and-formed beef jerky processes using commercial lactic acid bacteria starter cultures as pathogen surrogates. J Food Prot 72:1234-1247.

27. Brar PK, Danyluk M. 2019. Validation of *Enterococcus faecium* as a surrogate for *Salmonella* under different processing conditions for peanuts and pecans. Food Microbiol 80:9-17.

28. Brashears M, Jaroni D, Trimble J. 2003. Isolation, selection, and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157: H7 in cattle. J Food Prot 66:355-363.

29. Buege DR, Searls G, Ingham SC. 2006. Lethality of commercial whole-muscle beef jerky manufacturing processes against *Salmonella* serovars and *Escherichia coli* O157: H7. J Food Prot 69:2091-2099.

30. Callaway T, Edrington T, Anderson R, Harvey R, Genovese K, Kennedy C, Venn D, Nisbet D. 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim. Health Res. Rev. 9:217.

31. Cao Y, Fanning S, Proos S, Jordan K, Srikumar S. 2017. A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. Front Microbiol 8:1829.

32. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

33. Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N. 2016. Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. Nat. Microbiol. 2:1-6.

34. Carstens C, Salazar JK, Darkoh C. 2019. Multistate outbreaks of foodborne illness in the United States associated with fresh produce from 2010-2017. Front Microbiol 10:2667.

35. Caswell L, Fontenot J, Webb Jr K. 1975. Effect of processing method on pasturization and nitrogen components of broiler litter and on nitrogen utilization by sheep. J Anim Sci 40:750-759.

36. CDC 2016 https://www.cdc.gov/salmonella/pdf/CDC-Salmonella-Factsheet.pdf

37. Ceustermans A, De Clercq D, Aertsen A, Michiels C, Coosemans J, Ryckeboer J. 2007. Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. J Appl Microbiol 103:53-64.

38. Ceylan E, Bautista DA. 2015. Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for Salmonella for in-plant validation studies of low-moisture pet food products. J Food Prot 78:934-939.

39. Channaiah LH, Holmgren ES, Michael M, Sevart NJ, Milke D, Schwan CL, Krug M, Wilder A, Phebus RK, Thippareddi H. 2016. Validation of baking to control *Salmonella* serovars in hamburger bun manufacturing, and evaluation of *Enterococcus faecium* ATCC 8459 and *Saccharomyces cerevisiae* as nonpathogenic surrogate indicators. J Food Prot 79:544-552.

40. Chaturongakul S, Raengpradub S, Wiedmann M, Boor KJ. 2008. Modulation of stress and virulence in *Listeria monocytogenes*. Trends Microbiol 16:388-396.

41. Chen Z, Biswas S, Aminabadi P, Stackhouse JW, Jay-Russell MT, Pandey PK. 2019. Prevalence of *Escherichia coli* O157 and *Salmonella* spp. in solid bovine manure in California using real-time quantitative PCR. Lett Appl Microbiol 69:23-29.

42. Chen Z, Diao J, Dharmasena M, Ionita C, Jiang X, Rieck J. 2013. Thermal inactivation of desiccation-adapted *Salmonella* spp. in aged chicken litter. Appl Environ Microbiol 79:7013-7020. doi:10.1128/AEM.01969-13 [doi].

43. Chen Z, Jiang X. 2017. Microbiological safety of animal wastes processed by physical heat treatment: an alternative to eliminate human pathogens in biological soil amendments as recommended by the food safety modernization act. J Food Prot 80:392-405.

44. Chen Z, Kim J, Jiang X. 2018. Survival of *Escherichia coli* O157: H7 and *Salmonella* enterica in animal waste-based composts as influenced by compost type, storage condition and inoculum level. J Appl Microbiol 124:1311-1323.

45. Chen Z, Wang H, Ionita C, Luo F, Jiang X. 2015. Effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* spp. Appl Environ Microbiol 81:6883-6889. doi:10.1128/AEM.01876-15 [doi].

46. Cheng T, Li R, Kou X, Wang S. 2017. Influence of controlled atmosphere on thermal inactivation of *Escherichia coli* ATCC 25922 in almond powder. Food Microbiol 64:186-194.

47. Chinivasagam H, Redding M, Runge G, Blackall P. 2010. Presence and incidence of food-borne pathogens in Australian chicken litter. Br Poult Sci 51:311-318.

48. Clements D, Acuña-Maldonado L, Fisk C, Stoeckel D, Wall G, Woods K, Bihn E. 2019 FSMA Produce Safety Rule: documentation requirements for commercial soil amendment suppliers. https://ucanr.edu/sites/Small_Farms_/files/315707.pdf (Accessed on Feb. 2020)

49. Collazo C, Abadias M, Aguiló-Aguayo I, Alegre I, Chenoll E, Viñas I. 2017. Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7 against food-borne pathogens in vitro and on fresh-cut melon. LWT 85:301-308.

50. Collins E, Ogejo, Krometis, Howes. 2013. Evaluating the effects of pasteurization temperature and treatment duration on pathogen inactivation in separated liquid dairy manure, p. 1. *In* Anonymous 2013 Kansas City, Missouri, July 21-July 24, 2013. American Society of Agricultural and Biological Engineers.

51. Côté C, Massé DI, Quessy S. 2006. Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries. Bioresour Technol 97:686-691.

52. Cunault C, Burton C, Pourcher A. 2013. The impact of fouling on the process performance of the thermal treatment of pig slurry using tubular heat exchangers. J Environ Manage 117:253-262.

53. Cunault C, Pourcher A, Burton C. 2011. Using temperature and time criteria to control the effectiveness of continuous thermal sanitation of piggery effluent in terms of set microbial indicators. J Appl Microbiol 111:1492-1504.

54. Cytryn E. 2013. The soil resistome: the anthropogenic, the native, and the unknown. Soil Biol Biochem 63:18-23.

55. Danyluk MD, Zhao T, Doyle MP. 2007. Competitive inhibition bacteria of bovine origin against *Salmonella* serovars. J Food Prot 70:1804-1810.

56. Das G, Park S, Baek K. 2017. Diversity of endophytic bacteria in a fern species Dryopteris uniformis (Makino) Makino and evaluation of their antibacterial potential against five foodborne pathogenic bacteria. Foodborne Pathog. Dis. 14:260-268.

57. de Brito AM, Gagne S, Antoun H. 1995. Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. Appl Environ Microbiol 61:194-199. doi:10.1128/AEM.61.1.194-199.1995 [doi].

58. De Jong HK, Parry CM, van der Poll T, Wiersinga WJ. 2012. Host–pathogen interaction in invasive salmonellosis. PLOS pathog 8:e1002933.

59. de las Heras A, Cain RJ, Bielecka MK, Vazquez-Boland JA. 2011. Regulation of *Listeria* virulence: PrfA master and commander. Curr Opin Microbiol 14:118-127.

60. de Moraes MH, Chapin TK, Ginn A, Wright AC, Parker K, Hoffman C, Pascual DW, Danyluk MD, Teplitski M. 2016. Development of an avirulent *Salmonella*

surrogate for modeling pathogen behavior in pre- and postharvest environments. Appl Environ Microbiol 82:4100-4111. doi:10.1128/AEM.00898-16 [doi].

61. Deen B, Diez-Gonzalez F. 2019. Assessment of *Pediococcus acidilactici* ATCC 8042 as potential *Salmonella* surrogate for thermal treatments of toasted oats cereal and peanut butter. Food Microbiol 83:187-192.

62. Desneux J, Biscuit A, Picard S, Pourcher A. 2016. Fate of viable but non-culturable *Listeria monocytogenes* in pig manure microcosms. Front Microbiol 7:245.

63. Dharmasena M, Jiang X. 2018. Isolation of toxigenic *Clostridium difficile* from animal manure and composts being used as biological soil amendments. Appl Environ Microbiol 84:10.1128/AEM.00738-18. Print 2018 Aug 15. doi:e00738-18 [pii].

64. Dharmasena M, Wei T, Bridges Jr WC, Jiang X. 2019. Thermal resistance of Clostridium difficile endospores in dairy compost upon exposure to wet and dry heat treatments. J Appl Microbiol 127:274-283.

65. Donado-Godoy P, Gardner I, Byrne BA, Leon M, Perez-Gutierrez E, Ovalle M, Tafur M, Miller W. 2012. Prevalence, risk factors, and antimicrobial resistance profiles of Salmonella from commercial broiler farms in two important poultry-producing regions of Colombia. J Food Prot 75:874-883.

66. Dygico LK, O'Connor PM, Hayes M, Gahan CG, Grogan H, Burgess CM. 2019. *Lactococcus lactis* subsp. lactis as a natural anti-listerial agent in the mushroom industry. Food Microbiol 82:30-35.

67. Eblen DR, Annous BA, Sapers GM. 2005. Studies to select appropriate nonpathogenic surrogate *Escherichia coli* strains for potential use in place of *Escherichia coli* O157: H7 and *Salmonella* in pilot plant studies. J Food Prot 68:282-291.

68. Edrington TS, Fox WE, Callaway TR, Anderson RC, Hoffman DW, Nisbet DJ. 2009. Pathogen prevalence and influence of composted dairy manure application on antimicrobial resistance profiles of commensal soil bacteria. Foodborne Pathog. Dis. 6:217-224.

69. Eguale T. 2018. Non-typhoidal *Salmonella* serovars in poultry farms in central Ethiopia: prevalence and antimicrobial resistance. BMC Vet. Res. 14:217.

70. Elving J, Vinnerås B, Albihn A, Ottoson JR. 2014. Thermal treatment for pathogen inactivation as a risk mitigation strategy for safe recycling of organic waste in agriculture. J Environ Sci Heal B, 49:679-689.

71. Enache E, Kataoka A, Black DG, Napier CD, Podolak R, Hayman MM. 2015. Development of a dry inoculation method for thermal challenge studies in low-moisture foods by using talc as a carrier for *Salmonella* and a surrogate (*Enterococcus faecium*). J Food Prot 78:1106-1112.

72. Eng S, Pusparajah P, Ab Mutalib N, Ser H, Chan K, Lee L. 2015. *Salmonella*: a review on pathogenesis, epidemiology and antibiotic resistance. Front. Life Sci. 8:284-293.

73. Ercolini D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Methods 56:297-314.

74. Erickson M, Liao J, Ma L, Jiang X, Doyle M. 2014. Thermal and nonthermal factors affecting survival of *Salmonella* and *Listeria monocytogenes* in animal manure-based compost mixtures. J Food Prot 77:1512-1518.

75. Erickson MC, Smith C, Jiang X, Flitcroft ID, Doyle MP. 2015. Manure source and age affect survival of zoonotic pathogens during aerobic composting at sublethal temperatures. J Food Prot 78:302-310.

76. Fagbamila IO, Barco L, Mancin M, Kwaga J, Ngulukun SS, Zavagnin P, Lettini AA, Lorenzetto M, Abdu PA, Kabir J, Umoh J, Ricci A, Muhammad M. 2017. *Salmonella* serovars and their distribution in Nigerian commercial chicken layer farms. PLoS One 12:e0173097. doi:10.1371/journal.pone.0173097 [doi].

77. Falardeau J, Walji K, Haure M, Fong K, Taylor G, Ma Y, Smukler S, Wang S. 2018. Native bacterial communities and *Listeria monocytogenes* survival in soils collected from the Lower Mainland of British Columbia, Canada. Can J Microbiol 64:695-705.

78. Farber JM, Peterkin PI. 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol Rev 55:476-511.

79. Farzan A, Friendship R, Cook A, Pollari F. 2010. Occurrence of *Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, *Escherichia coli* O157 and *Listeria monocytogenes* in swine. Zoonoses and public health 57:388-396.

80. FDA 2018. 21CFR112.55. Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=112.55 (Accessed August 2, 2019).

81. FDA CFR 2019. §205.203 Soil fertility and crop nutrient management practice standard. Available at: https://www.ecfr.gov/cgi-bin/text-idx?SID=5b54f57ac35925f92ec258714e838791&mc=true&node=se7.3.205_1203&rgn=div8 (Accessed August 2, 2020).

82. FDA FSMA 2019 https://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm334114.htm

83. Fett WF. 2006. Inhibition of *Salmonella enterica* by plant-associated pseudomonads in vitro and on sprouting alfalfa seed. J Food Prot 69:719-728.

84. Fira D, Dimkić I, Berić T, Lozo J, Stanković S. 2018. Biological control of plant pathogens by *Bacillus* species. J Biotechnol 285:44-55.

85. Fittipaldi M, Nocker A, Codony F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Methods 91:276-289.

86. Flockhart L, Pintar K, Cook A, McEwen S, Friendship R, Kelton D, Pollari F. 2017. Distribution of *Salmonella* in humans, production animal operations and a watershed in a FoodNet Canada sentinel site. Zoonoses Public Health 64:41-52.

87. Fox EM, Solomon K, Moore JE, Wall PG, Fanning S. 2014. Phylogenetic profiles of in-house microflora in drains at a food production facility: comparison and biocontrol implications of *Listeria*-positive and -negative bacterial populations. Appl Environ Microbiol 80:3369-3374. doi:10.1128/AEM.00468-14 [doi].

88. Franke-Whittle IH, Klammer SH, Insam H. 2005. Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J Microbiol Methods 62:37-56.

89. Freitag NE, Port GC, Miner MD. 2009. *Listeria monocytogenes* from saprophyte to intracellular pathogen. Nat. Rev. Microbiol. 7:623-628.

90. Fudge J, Dunn M, Pike O, Robison R, Steele F. 2016. The isolation and identification of *Pantoea dispersa* strain JFS as a non-pathogenic surrogate for *Salmonella* Typhimurium phage type 42 in flour. Int J Food Microbiol 219:1-6.

91. Gascho GJ, Hubbard RK. 2006. Long-term impact of broiler litter on chemical properties of a Coastal Plain soil. J Soil Water Conserv 61:65-74.

92. Gelinski, Jane Mary Lafayette Neves, Baratto CM, Casagrande M, de Oliveira TP, Megiolaro F, de Martini Soares, Fabiana Andreia S, de Souza, Endler Marcel Borges, Vicente VA, Fonseca GG. 2019. Control of pathogens in fresh pork sausage by inclusion of *Lactobacillus sakei* BAS0117. Can J Microbiol 65:831-841.

93. Gensler CA, Brown SR, Aljasir SF, D'amico DJ. 2020. Compatibility of commercially produced protective cultures with common cheesemaking cultures and their antagonistic effect on foodborne pathogens. J Food Prot 83:1010-1019.

94. Ghaly A, Alhattab M. 2013. Drying poultry manure for pollution potential reduction and production of organic fertilizer. Am. J. Environ. Sci. 9:88-102.

95. Gholipour S, Nikaeen M, Farhadkhani M, Nikmanesh B. 2020. Survey of *Listeria monocytogenes* contamination of various environmental samples and associated health risks. Food Control 108:106843.

96. Gilbert JA, Hughes M. 2011. Gene expression profiling: metatranscriptomics, p. 195-205. *In* Anonymous High-Throughput next Generation Sequencing. Springer.

97. Goberna M, Podmirseg S, Waldhuber S, Knapp B, García C, Insam H. 2011. Pathogenic bacteria and mineral N in soils following the land spreading of biogas digestates and fresh manure. Appl Soil Ecol. 49:18-25.

98. Gong CM. 2007. Microbial safety control of compost material with cow dung by heat treatment. J. Environ. Sci. 19:1014-1019.

99. Graham JP, Price LB, Evans SL, Graczyk TK, Silbergeld EK. 2009. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Sci Total Environ 407:2701-2710.

100. Grewal S, Sreevatsan S, Michel Jr FC. 2007. Persistence of *Listeria* and *Salmonella* during swine manure treatment. Compost Sci Util 15:53-62.

101. Grewal SK, Rajeev S, Sreevatsan S, Michel FC,Jr. 2006. Persistence of *Mycobacterium avium* subsp. paratuberculosis and other zoonotic pathogens during simulated composting, manure packing, and liquid storage of dairy manure. Appl Environ Microbiol 72:565-574. doi:72/1/565 [pii].

102. Gueimonde M, Jalonen L, He F, Hiramatsu M, Salminen S. 2006. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. Food Res Int 39:467-471.

103. Guerra M, McLauchlin J, Bernardo F. 2001. *Listeria* in ready-to-eat and unprocessed foods produced in Portugal. Food Microbiol 18:423-429.

104. Guévremont E, Lamoureux L, Généreux M, Côté C. 2017. Irrigation water sources and time intervals as variables on the presence of *Campylobacter* spp. and *Listeria monocytogenes* on romaine lettuce grown in muck soil. J Food Prot 80:1182-1187.

105. Gurtler JB, Doyle MP, Erickson MC, Jiang X, Millner P, Sharma M. 2018. Composting to inactivate foodborne pathogens for crop soil application: a review. J Food Prot 81:1821-1837.

106. Gurtler JB. 2017. Pathogen decontamination of food crop soil: a review. J Food Prot 80:1461-1470.

107. Habing GG, Lombard JE, Kopral CA, Dargatz DA, Kaneene JB. 2012. Farm-level associations with the shedding of *Salmonella* and antimicrobial-resistant *Salmonella* in US dairy cattle. Foodborne Pathog. Dis. 9:815-821.

108. Hafez YM, Sobeih AM, Mansour NM. 2019. Pivotal role of *lactobacillus* strains in improvement of soft cheese quality and inhibiting the growth of harmful and dangerous bacterial pathogens. Slov Vet Res. 56:.

109. Harp E, Gilliland S. 2003. Evaluation of a select strain of *Lactobacillus delbrueckii* subsp. lactis as a biological control agent for pathogens on fresh-cut vegetables stored at 7°C. J Food Prot 66:1013-1018.

110. Harris LJ, Bender J, Bihn EA, Blessington T, Danyluk MD, Delaquis P, Goodridge L, Ibekwe AM, Ilic S, Kniel K. 2012. A framework for developing

research protocols for evaluation of microbial hazards and controls during production that pertain to the quality of agricultural water contacting fresh produce that may be consumed raw. J Food Prot 75:2251-2273.

111. Hassen A, Belguith K, Jedidi N, Cherif A, Cherif M, Boudabous A. 2001. Microbial characterization during composting of municipal solid waste. Bioresour Technol 80:217-225.

112. Heise J, Nega M, Alawi M, Wagner D. 2016. Propidium monoazide treatment to distinguish between live and dead methanogens in pure cultures and environmental samples. J Microbiol Methods 121:11-23.

113. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8:15-25.

114. Himathongkham S, Bahari S, Riemann H, Cliver D. 1999. Survival of *Escherichia coli* O157: H7 and Salmonella typhimurium in cow manure and cow manure slurry. FEMS Microbiol Lett 178:251-257.

115. Himathongkham S, Nuanualsuwan S, Riemann H. 1999. Survival of *Salmonella* enteritidis and *Salmonella* typhimurium in chicken manure at different levels of water activity. FEMS Microbiol Lett 172:159-163.

116. Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G. 2006. *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. J Environ Qual 35:1170-1180.

117. Hoszowski A, Wasyl D. 2001. *Salmonella* spp. found in wastes, sewage sludge, compost and their antimicrobial resistance. Bulletin-veterinary institute in pulawy 45:163-170.

118. Hu M, Gurtler JB. 2017. Selection of surrogate bacteria for use in food safety challenge studies: a review. J Food Prot 80:1506-1536.

119. Hueso S, García C, Hernández T. 2012. Severe drought conditions modify the microbial community structure, size and activity in amended and unamended soils. Soil Biol Biochem 50:167-173.

120. Hugerth LW, Andersson AF. 2017. Analyzing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. Front. Microbiol. 8:1561.

121. Hutchison M, Walters L, Avery S, Synge B, Moore A. 2004. Levels of zoonotic agents in British livestock manures. Lett Appl Microbiol 39:207-214.

122. Hutchison ML, Walters LD, Avery SM, Munro F, Moore A. 2005. Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. Appl Environ Microbiol 71:1231-1236. doi:71/3/1231 [pii].

123. Hutchison ML, Walters LD, Moore A, Crookes KM, Avery SM. 2004. Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. Appl Environ Microbiol 70:5111-5118. doi:10.1128/AEM.70.9.5111-5118.2004 [doi].

124. Hutchison ML, Walters LD, Moore T, Thomas DJ, Avery SM. 2005. Fate of pathogens present in livestock wastes spread onto fescue plots. Appl Environ Microbiol 71:691-696. doi:71/2/691 [pii].

125. Iglesias M, Abadias M, Anguera M, Viñas I. 2018. Efficacy of *Pseudomonas graminis* CPA-7 against *Salmonella* spp. and *Listeria monocytogenes* on fresh-cut pear and setting up of the conditions for its commercial application. Food Microbiol 70:103-112.

126. International Code of Nomenclature of Prokaryotes (ICNP) https://lpsn.dsmz.de/text/numbers Accessed on Mar. 2020

127. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. J Food Prot 67:1365-1370.

128. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. Appl Environ Microbiol 70:2497-2502.

129. Jay-Russell MT, Madigan JE, Bengson Y, Madigan S, Hake A, Foley JE, Byrne BA. 2014. *Salmonella Oranienburg* isolated from horses, wild turkeys and an edible home garden fertilized with raw horse manure. Zoonoses Public Health 61:64-71.

130. Jeong S, Marks BP, James MK. 2017. Comparing thermal process validation methods for Salmonella inactivation on almond kernels. J Food Prot 80:169-176.

131. Jeong S, Marks BP, Ryser ET. 2011. Quantifying the performance of *Pediococcus* sp.(NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. J Food Prot 74:603-609.

132. Ji ZH, Ren WZ, Gao W, Hao Y, Gao W, Chen J, Quan FS, Hu JP, Yuan B. 2017. Analyzing the innate immunity of NIH hairless mice and the impact of gut microbial polymorphisms on *Listeria monocytogenes* infection. Oncotarget. 8:106222-106232. doi:10.18632/oncotarget.22051 [doi].

133. Jiang X, Islam M, Morgan J, Doyle MP. 2004. Fate of *Listeria monocytogenes* in bovine manure-amended soil. J Food Prot 67:1676-1681.

134. Jin T, Zhang H, Boyd G, Tang J. 2008. Thermal resistance of *Salmonella* enteritidis and *Escherichia coli* K12 in liquid egg determined by thermal-death-time disks. J Food Eng 84:608-614.

135. KABAN G, KAYA M, LÜCKE F. 2010. The effect of *Lactobacillus Sakei* on the behavior of *Listeria monocytogenes* on sliced Bologna-type sausages. J Food Saf 30:889-901.

136. KagambÃga A, Thibodeau A, Trinetta V, Soro DK, Sama FN, Bako Ã, Bouda CS, Wereme NâDiaye A, Fravalo P, Barro N. 2018. *Salmonella* spp. and *Campylobacter* spp. in poultry feces and carcasses in Ouagadougou, Burkina Faso. Food Sci Nutr 6:1601-1606.

137. Karami S, Roayaei M, Hamzavi H, Bahmani M, Hassanzad-Azar H, Leila M, Rafieian-Kopaei M. 2017. Isolation and identification of probiotic *Lactobacillus* from local dairy and evaluating their antagonistic effect on pathogens. Int J Pharm Investig 7:137-141. doi:10.4103/jphi.JPHI_8_17 [doi].

138. Khan N, Clark I, Sánchez-Monedero MA, Shea S, Meier S, Qi F, Kookana RS, Bolan N. 2016. Physical and chemical properties of biochars co-composted with biowastes and incubated with a chicken litter compost. Chemosphere 142:14-23.

139. Kharel K, Yemmireddy VK, Graham CJ, Prinyawiwatkul W, Adhikari A. 2018. Hot water treatment as a kill-step to inactivate *Escherichia coli* O157: H7, *Salmonella enterica*, *Listeria monocytogenes* and *Enterococcus faecium* on in-shell pecans. LWT 97:555-560.

140. Kim J, Diao J, Shepherd MW,Jr, Singh R, Heringa SD, Gong C, Jiang X. 2012. Validating thermal inactivation of *Salmonella* spp. in fresh and aged chicken litter. Appl Environ Microbiol 78:1302-1307. doi:10.1128/AEM.06671-11 [doi].

141. Kim J, Shepherd MW, Jiang X. 2009. Evaluating the effect of environmental factors on pathogen regrowth in compost extract. Microb Ecol 58:498-508.

142. Kim S, Bang J, Kim H, Beuchat LR, Ryu J. 2013. Inactivation of *Escherichia coli* O157: H7 on stainless steel upon exposure to *Paenibacillus polymyxa* biofilms. Int J Food Microbiol 167:328-336.

143. Kim W, Choi SY, Han I, Cho SK, Lee Y, Kim S, Kang B, Choi O, Kim J. 2020. Inhibition of *Salmonella enterica* growth by competitive exclusion during early

alfalfa sprout development using a seed-dwelling Erwinia persicina strain EUS78. Int J Food Microbiol 312:108374.

144. Kim Y, Kim H, Beuchat LR, Ryu J. 2018. Development of non-pathogenic bacterial biofilms on the surface of stainless steel which are inhibitory to *Salmonella enterica*. Food Microbiol 69:136-142.

145. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:e1-e1.

146. Köhl J, Kolnaar R, Ravensberg WJ. 2019. Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Front. Plant Sci. 10:845.

147. Kondrotiene K, Kasnauskyte N, Serniene L, Gölz G, Alter T, Kaskoniene V, Maruska AS, Malakauskas M. 2018. Characterization and application of newly isolated nisin producing *Lactococcus* lactis strains for control of *Listeria monocytogenes* growth in fresh cheese. LWT 87:507-514.

148. Kopit LM, Kim EB, Siezen RJ, Harris LJ, Marco ML. 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. Appl Environ Microbiol 80:1899-1909. doi:10.1128/AEM.03859-13 [doi].

149. La Ragione RM, Woodward MJ. 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet Microbiol 94:245-256.

150. Lalloo R, Moonsamy G, Ramchuran S, Görgens J, Gardiner N. 2010. Competitive exclusion as a mode of action of a novel *Bacillus cereus* aquaculture biological agent. Lett Appl Microbiol 50:563-570.

151. Larney FJ, Blackshaw RE. 2003. Weed seed viability in composted beef cattle feedlot manure. J Environ Qual 32:1105-1113.

152. Larney FJ, Yanke LJ, Miller JJ, McAllister TA. 2003. Fate of coliform bacteria in composted beef cattle feedlot manure. J Environ Qual 32:1508-1515.

153. Li R, Tun HM, Jahan M, Zhang Z, Kumar A, Fernando WD, Farenhorst A, Khafipour E. 2017. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. Sci. Rep. 7:1-11.

154. Li X, Payne J, Santos F, Levine J, Anderson K, Sheldon B. 2007. *Salmonella* populations and prevalence in layer feces from commercial high-rise houses and characterization of the Salmonella isolates by serotyping, antibiotic resistance analysis, and pulsed field gel electrophoresis. Poult Sci 86:591-597.

155. Liao C. 2009. Control of foodborne pathogens and soft-rot bacteria on bell pepper by three strains of bacterial antagonists. J Food Prot 72:85-92.

156. Liu B, Li Y, Zhang X, Wang J, Gao M. 2014. Combined effects of chlortetracycline and dissolved organic matter extracted from pig manure on the functional diversity of soil microbial community. Soil Biol Biochem 74:148-155.

157. Liu L, Liang M, Li L, Sun L, Xu Y, Gao J, Wang L, Hou Y, Huang S. 2018. Synergistic effects of the combined application of *Bacillus subtilis* H158 and strobilurins for rice sheath blight control. Biol. Control 117:182-187.

158. Liu S, Rojas RV, Gray P, Zhu M, Tang J. 2018. Enterococcus faecium as a *Salmonella* surrogate in the thermal processing of wheat flour: Influence of water activity at high temperatures. Food Microbiol 74:92-99.

159. Liu S, Tang J, Tadapaneni RK, Yang R, Zhu MJ. 2018. Exponentially Increased Thermal resistance of *Salmonella* spp. and *Enterococcus faecium* at reduced water activity. Appl Environ Microbiol 84:10.1128/AEM.02742-17. Print 2018 Apr 15. doi:e02742-17 [pii].

160. Loebenstein G, Carr J. Natural and Engineered Resistance to Plant Viruses. Academic Press; 2009 Nov 3.

161. López-García A, Pineda-Quiroga C, Atxaerandio R, Pérez A, Hernández I, García-Rodríguez A, González-Recio O. 2018. Comparison of Mothur and QIIME for the analysis of rumen microbiota composition based on 16S rRNA amplicon sequences. Front. Microbiol. 9:3010.

162. Lung A, Lin C, Kim J, Marshall M, Nordstedt R, Thompson N, Wei C. 2001. Destruction of *Escherichia coli* O157: H7 and *Salmonella* enteritidis in cow manure composting. J Food Prot 64:1309-1314.

163. Ma L, Kornacki JL, Zhang G, Lin C, Doyle MP. 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. J Food Prot 70:952-957.

164. Ma S, Fang C, Sun X, Han L, He X, Huang G. 2018. Bacterial community succession during pig manure and wheat straw aerobic composting covered with a semi-permeable membrane under slight positive pressure. Bioresour Technol 259:221-227.

165. Maurice Bilung L, Sin Chai L, Tahar AS, Ted CK, Apun K. 2018. Prevalence, genetic heterogeneity, and antibiotic resistance profile of listeria spp. and *Listeria monocytogenes* at farm level: a highlight of ERIC-and BOX-PCR to reveal genetic diversity. Biomed Res. Int. 2018:.

166. McCarthy G, Lawlor PG, Gutierrez M, Sullivan L, Murphy A, Zhan X, Gardiner GE. 2015. An assessment of *Salmonella* survival in pig manure and its separated solid and liquid fractions during storage. J. Environ. Sci. Health B 50:135-145.

167. McGarvey, J.A., Hnasko, R.M., Stanker, L.H. and Gorski, L.A., US Department of Agriculture, 2019. Use of phyllosphere associated lactic acid bacteria as biocontrol agents to reduce bacterial growth on fresh produce. U.S. Patent 10,264,808.

168. Meneghine AK, Nielsen S, Varani AM, Thomas T, Carareto Alves LM. 2017. Metagenomic analysis of soil and freshwater from zoo agricultural area with organic fertilization. PloS one 12:e0190178.

169. Messer JW, Lovett J, Murthy GK, Wehby AJ, Schafer ML, Read Jr RB. 1971. An Assessment of some public health problems resulting from feeding poultry litter to animals.: Microbiological and Chemical Parameters. Poult Sci 50:874-881.

170. Miller C, Heringa S, Kim J, Jiang X. 2013. Analyzing indicator microorganisms, antibiotic resistant *Escherichia coli*, and regrowth potential of foodborne pathogens in various organic fertilizers. Foodborne Pathog. Dis.10:520-527.

171. Moran MA. 2009. Metatranscriptomics: eavesdropping on complex microbial communities. Microbe 4:7.

172. Morgan XC, Huttenhower C. 2012. Human microbiome analysis. PLoS Comput Biol 8:e1002808.

173. Moslehi-Jenabian S, Vogensen FK, Jespersen L. 2011. The quorum sensing luxS gene is induced in *Lactobacillus acidophilus* NCFM in response to *Listeria monocytogenes*. Int J Food Microbiol 149:269-273.

174. Moussavi M, Frelka JC, Hildebrandt IM, Marks BP, Harris LJ. 2020. Thermal resistance of foodborne pathogens and *Enterococcus faecium* NRRL B-2354 on Inoculated Pistachios. J Food Prot Feb 21.

175. Mulero-Aparicio A, Trapero A, LÓPEZ-ESCUDERO FJ. 2020. A non-pathogenic strain of *Fusarium oxysporum* and grape marc compost control Verticillium wilt of olive. Phytopathol Mediterr 59:159-167.

176. Muñoz N, Sonar CR, Bhunia K, Tang J, Barbosa-Cánovas GV, Sablani SS. 2019. Use of protective culture to control the growth of *Listeria monocytogenes* and *Salmonella typhimurium* in ready-to-eat cook-chill products. Food Control 102:81-86.

177. NÃ³grÃ¡dy N, Kardos G, Bistyak A, TurcsÃ¡nyi I, MÃ-szÃ¡ros J, GalÃintai Z, JuhÃisz A, Samu P, Kaszanyitzky J, PÃiszti J. 2008. Prevalence and

characterization of *Salmonella infantis* isolates originating from different points of the broiler chicken human food chain in Hungary. Int J Food Microbiol 127:162-167.

178. National Organic Program (NOP). (2006). National Organic Standards Board Crops Committee Recommendation for guidance use of compost, vermicompost, processed manure, and compost teas.

179. Nega A. 2014. Review on concepts in biological control of plant pathogens. Journal of Biology, Agriculture and Healthcare 4:33-54.

180. Neher DA, Weicht TR, Bates ST, Leff JW, Fierer N. 2013. Changes in bacterial and fungal communities across compost recipes, preparation methods, and composting times. PloS one 8:e79512.

181. Ngodigha E, Owen O. 2009. Evaluation of the bacteriological characteristics of poultry litter as feedstuff for cattle. SRE 4:188-190.

182. Nicholson FA, Groves SJ, Chambers BJ. 2005. Pathogen survival during livestock manure storage and following land application. Bioresour Technol 96:135-143.

183. Nocker A, Burr M, Camper AK. 2007. Genotypic microbial community profiling: a critical technical review. Microb Ecol 54:276-289.

184. Nurmi E, Nuotio L, Schneitz C. 1992. The competitive exclusion concept: development and future. Int J Food Microbiol 15:237-240.

185. Obaidat MM, Stringer AP. 2019. Prevalence, molecular characterization, and antimicrobial resistance profiles of *Listeria monocytogenes*, *Salmonella enterica*,

and *Escherichia coli* O157: H7 on dairy cattle farms in Jordan. J Dairy Sci 102:8710-8720.

186. O'Bryan CA, Crandall PG, Martin EM, Griffis CL, Johnson MG. 2006. Heat resistance of *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* 0157: H7, and *Listeria innocua* M1, a potential surrogate for *Listeria monocytogenes*, in meat and poultry: A review. J Food Sci 71:R23-R30.

187. Okike IO, Lamont RF, Heath PT. 2013. Do we really need to worry about *Listeria* in newborn infants? Pediatr Infect Dis J 32:405-406. doi:10.1097/INF.0b013e3182867fa0 [doi].

188. Orji MU, Onuigbo HC, Mbata TI. 2005. Isolation of *Salmonella* from poultry droppings and other environmental sources in Awka, Nigeria. International J. Infect. Dis. 9:86-89.

189. Paliy O, Shankar V. 2016. Application of multivariate statistical techniques in microbial ecology. Mol Ecol 25:1032-1057.

190. Pandey P, Chiu C, Miao M, Wang Y, Settles M, Del Rio NS, Castillo A, Souza A, Pereira R, Jeannotte R. 2018. 16S rRNA analysis of diversity of manure microbial community in dairy farm environment. PloS one 13:e0190126.

191. Pandey PK, Cao W, Wang Y, Vaddella V, Castillo AR, Souza A, Del Rio NS. 2016. Simulating the effects of mesophilic anaerobic and aerobic digestions, lagoon system, and composting on pathogen inactivation. Ecol Eng 97:633-641.

192. Pastrana AM, Basallote-Ureba MJ, Aguado A, Akdi K, Capote N. 2016. Biological control of strawberry soil-borne pathogens *Macrophomina phaseolina*

and *Fusarium solani*, using *Trichoderma asperellum* and *Bacillus* spp. Phytopathol Mediterr 109-120.

193. Phanâ-Thien K, Metaferia MH, Bell TL, Bradbury MI, Sassi HP, Van Ogtrop FF, Suslow TV, McConchie R. 2020. Effect of soil type and temperature on survival of *Salmonella enterica* in poultry manure-amended soils. Lett Appl Microbiol .

194. Poirier D, Sanders T, Davis J. 2014. *Salmonella* surrogate reduction using industrial peanut dry roasting parameters. Peanut Sci. 41:72-84.

195. Pornsukarom S, Thakur S. 2016. Assessing the impact of manure application in commercial swine farms on the transmission of antimicrobial resistant *Salmonella* in the environment. PLoS One 11:e0164621. doi:10.1371/journal.pone.0164621 [doi].

196. Postma J, Nijhuis EH. 2019. *Pseudomonas chlororaphis* and organic amendments controlling Pythium infection in tomato. Eur J Plant Pathol 154:91-107.

197. Postma, J., Os, E.V. and Bonants, P.J.M., 2008. Pathogen detection and management strategies in soilless plant growing systems In: Raviev, M., Lieth, JH (eds.) Soilless Culture: Theory and Practice.

198. Powell J, Witthuhn R, Todorov S, Dicks L. 2007. Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. Int Dairy J 17:190-198.

199. Prazak AM, Murano EA, Mercado I, Acuff GR. 2002. Prevalence of *Listeria monocytogenes* during production and postharvest processing of cabbage. J Food Prot 65:1728-1734.

200. Pulschen AA, Bendia AG, Fricker AD, Pellizari VH, Galante D, Rodrigues F. 2017. Isolation of uncultured bacteria from antarctica using long incubation periods and low nutritional media. Front. Microbiol. 8:1346.

201. Puri A, Dudley EG. 2010. Influence of indigenous eukaryotic microbial communities on the reduction of *Escherichia coli* O157: H7 in compost slurry. FEMS Microbiol Lett 313:148-154.

202. Qian G, Hu B, Jiang Y, Liu F. 2009. Identification and characterization of *Lysobacter enzymogenes* as a biological control agent against some fungal pathogens. Agr Sci China 8:68-75.

203. Qiu M, Zhang R, Xue C, Zhang S, Li S, Zhang N, Shen Q. 2012. Application of bio-organic fertilizer can control *Fusarium wilt* of cucumber plants by regulating microbial community of rhizosphere soil. Biol Fertility Soils 48:807-816.

204. Rachon G, Peñaloza W, Gibbs PA. 2016. Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. Int J Food Microbiol 231:16-25.

205. Redemann MA, Brar J, Niebuhr SE, Lucia LM, Acuff GR, Dickson JS, Singh M. 2018. Evaluation of thermal process lethality for non-pathogenic *Escherichia coli* as a surrogate for *Salmonella* in ground beef. LWT 90:290-296.

206. Ren X, Zhang N, Cao M, Wu K, Shen Q, Huang Q. 2012. Biological control of tobacco black shank and colonization of tobacco roots by a *Paenibacillus polymyxa* strain C5. Biol Fertility Soils 48:613-620.

207. Riesenfeld CS, Schloss PD, Handelsman J. 2004. Metagenomics: genomic analysis of microbial communities. Annu Rev Genet 38:525-552.

208. Ruiz-Espinoza JE, Méndez-Contreras JM, Alvarado-Lassman A, Martínez-Delgadillo SA. 2012. Effect of low temperature thermal pre-treatment on the solubilization of organic matter, pathogen inactivation and mesophilic anaerobic digestion of poultry sludge. J. Environ. Sci. Health A 47:1795-1802.

209. Rynk R, Van de Kamp M, Willson GB, Singley ME, Richard TL, Kolega JJ, Gouin FR, Laliberty L, Kay D, Murphy D. 1992. On-Farm Composting Handbook (NRAES 54). Northeast Regional Agricultural Engineering Service (NRAES).

210. Samelis J, Giannou E, Pappa EC, Bogović-Matijašić B, Lianou A, Parapouli M, Drainas C. 2017. Behavior of artificial listerial contamination in model Greek Graviera cheeses manufactured with the indigenous nisin A-producing strain *Lactococcus lactis* subsp. cremoris M104 as costarter culture. J Food Saf 37:e12326.

211. Sánchez ÓJ, Ospina DA, Montoya S. 2017. Compost supplementation with nutrients and microorganisms in composting process. Waste Manage. Nov 1;69:136-53.

212. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15. doi:10.3201/eid1701.P11101 [doi].

213. Schierstaedt J, Jechalke S, Nesme J, Neuhaus K, Sørensen SJ, Grosch R, Smalla K, Schikora A. 2020. *Salmonella* persistence in soil depends on reciprocal interactions with indigenous microorganisms. Environ Microbiol.

214. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537-7541. doi:10.1128/AEM.01541-09 [doi].

215. Schneitz C. 2005. Competitive exclusion in poultry—30 years of research. Food control 16:657-667.

216. Schultze DM, Couto R, Temelli F, McMullen LM, Gänzle M. 2020. Lethality of high-pressure carbon dioxide on Shiga toxin-producing *Escherichia coli*, *Salmonella* and surrogate organisms on beef jerky. Int J Food Microbiol 321:108550.

217. Semenov AV, van Overbeek L, van Bruggen AH. 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. Appl Environ Microbiol 75:3206-3215. doi:10.1128/AEM.01791-08 [doi].

218. Shah MK, Asa G, Sherwood J, Graber K, Bergholz TM. 2017. Efficacy of vacuum steam pasteurization for inactivation of *Salmonella* PT 30, *Escherichia*

coli O157: H7 and *Enterococcus faecium* on low moisture foods. Int J Food Microbiol 244:111-118.

219. Shah MK, Bradshaw R, Nyarko E, Millner PD, Neher D, Weicht T, Bergholz TM, Sharma M. 2019. Survival and growth of wild-type and rpoS-deficient *Salmonella* Newport strains in soil extracts prepared with heat-treated poultry pellets. J Food Prot 82:501-506.

220. Shah N, Tang H, Doak TG, Ye Y. 2011. Comparing bacterial communities inferred from 16S rRNA gene sequencing and shotgun metagenomics, p. 165-176. In Anonymous Biocomputing 2011. World Scientific.

221. Shakya M, Lo C, Chain PS. 2019. Advances and challenges in Metatranscriptomic analysis. Front. Genet. 10:904.

222. Sharifazizi M, Harighi B, Sadeghi A. 2017. Evaluation of biological control of *Erwinia amylovora*, causal agent of fire blight disease of pear by antagonistic bacteria. Biol. Control 104:28-34.

223. Sheng L, Shen X, Benedict C, Su Y, Tsai H, Schacht E, Kruger CE, Drennan M, Zhu M. 2019. Microbial safety of dairy manure fertilizer application in raspberry production. Front. Microbiol. 10:.

224. Shepherd Jr M, Liang P, Jiang X, Doyle M, Erickson M. 2010. Microbiological analysis of composts produced on South Carolina poultry farms. J Appl Microbiol 108:2067-2076.

225. Shi S, Qi Z, Sheng T, Tu J, Shao Y, Qi K. 2019. Antagonistic trait of *Lactobacillus reuteri* S5 against *Salmonella* enteritidis and assessment of its potential probiotic characteristics. Microb Pathog 137:103773.

226. Simon K, Simon V, Rosenzweig R, Barroso R, Gillmor-Kahn M. 2018. *Listeria*, Then and Now: A call to reevaluate patient teaching based on analysis of US federal databases, 1998-2016. J Midwifery Womens Health 63:301-308.

227. Singh R, Jiang X, Luo F. 2010. Thermal inactivation of heat-shocked *Escherichia coli* O157: H7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. J Food Prot 73:1633-1640.

228. Singh R, Jiang X. 2012. Thermal inactivation of acid-adapted *Escherichia coli* O157: H7 in dairy compost. Foodborne Pathog. Dis. 9:741-748.

229. Slana I, Pribylova R, Kralova A, Pavlik I. 2011. Persistence of *Mycobacterium avium* subsp. paratuberculosis at a farm-scale biogas plant supplied with manure from paratuberculosis-affected dairy cattle. Appl Environ Microbiol 77:3115-3119. doi:10.1128/AEM.02407-10 [doi].

230. Smolińska U, Kowalska B. 2018. Biological control of the soil-borne fungal pathogen *Sclerotinia sclerotiorum*—a review. J Plant Pathol 100:1-12.

231. Son H, Park S, Beuchat LR, Kim H, Ryu J. 2016. Inhibition of *Staphylococcus aureus* by antimicrobial biofilms formed by competitive exclusion microorganisms on stainless steel. Int J Food Microbiol 238:165-171.

232. Strawn LK, Fortes ED, Bihn EA, Nightingale KK, Grohn YT, Worobo RW, Wiedmann M, Bergholz PW. 2013. Landscape and meteorological factors

affecting prevalence of three food-borne pathogens in fruit and vegetable farms. Appl Environ Microbiol 79:588-600. doi:10.1128/AEM.02491-12 [doi].

233. Strawn LK, Grohn YT, Warchocki S, Worobo RW, Bihn EA, Wiedmann M. 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. Appl Environ Microbiol 79:7618-7627. doi:10.1128/AEM.02831-13 [doi].

234. Stringfellow K, Caldwell D, Lee J, Byrd A, Carey J, Kessler K, McReynolds J, Bell A, Stipanovic R, Farnell M. 2010. Pasteurization of chicken litter with steam and quicklime to reduce *Salmonella* Typhimurium. J Appl Poult Res 19:380-386.

235. Szala B, Paluszak Z, Motyl I. 2012. Antagonistic effect of lactic acid bacteria on *Salmonella* Senftenberg in mixed cultures. Pol.J.Environ.Stud 21:1399-1403.

236. Tamanini R, Beloti V, da Silva, Livia Cavaletti Correa, da Angela HL, Yamada AK, Battaglini APP, Fagnani R, Monteiro AA. 2012. Antagonistic activity against *Listeria monocytogenes* and *Escherichia coli* from lactic acid bacteria isolated from raw milk. Semina: Ciências Agrárias 33:1877-1886.

237. Tan X, Chung T, Chen Y, Macarisin D, LaBorde L, Kovac J. 2019. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. Microbiome 7:115.

238. Thunberg RL, Tran TT, Bennett RW, Matthews RN, Belay N. 2002. Microbial evaluation of selected fresh produce obtained at retail markets. J Food Prot 65:677-682.

239. Topalcengiz Z, Spanninger PM, Jeamsripong S, Persad AK, Buchanan RL, Saha J, LeJeune J, Jay-Russell MT, Kniel KE, Danyluk MD. 2020. Survival of *Salmonella* in various wild animal feces that may contaminate produce. J Food Prot 83:651-660.

240. Toth J, Aceto H, Rankin S, Dou Z. 2011. Survival characteristics of *Salmonella enterica* serovar Newport in the dairy farm environment. J Dairy Sci 94:5238-5246.

241. Trias R, Badosa E, Montesinos E, Bañeras L. 2008. Bioprotective *Leuconostoc* strains against *Listeria monocytogenes* in fresh fruits and vegetables. Int J Food Microbiol 127:91-98.

242. Trimble LM, Alali WQ, Gibson KE, Ricke SC, Crandall P, Jaroni D, Berrang M, Habteselassie MY. 2013. Prevalence and concentration of *Salmonella* and *Campylobacter* in the processing environment of small-scale pastured broiler farms. Poult Sci 92:3060-3066.

243. Tsai H, Ballom KF, Xia S, Tang J, Marks BP, Zhu M. 2019. Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during cocoa powder thermal processing. Food Microbiol 82:135-141.

244. Turhan EU, Erginkaya Z, Uney MH, Ozer EA. 2017. Inactivation effect of probiotic biofilms on growth of *Listeria monocytogenes*. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 23:.

245. U. S. Environmental Protection Agency (USEPA) 2018, Ed. Use of Composting for Biosolids Management. https://www.epa.gov/sites/production/files/2018-11/documents/use-composing-biosolids-management.pdf

246. USDA 2018 Analysis of economic impacts- Standards for the growing, harvesting, packing and holding of produce for human consumption. https://www.ers.usda.gov/webdocs/publications/89749/eib-195.pdf?v=0

247. Vandeplas S, Marcq C, Dubois Dauphin R, Beckers Y, Thonart P, Théwis A. 2008. Contamination of poultry flocks by the human pathogen *Campylobacter* spp. and strategies to reduce its prevalence at the farm level. Biotechnologie, Agronomie, Société et Environnement 12:317-334.

248. Vasan A, Geier R, Ingham SC, Ingham BH. 2014. Thermal tolerance of O157 and non-O157 Shiga toxigenic strains of *Escherichia coli*, *Salmonella*, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. J Food Prot 77:1501-1511.

249. Verma T, Wei X, Lau SK, Bianchini A, Eskridge KM, Subbiah J. 2018. Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* during extrusion of low-moisture food. J Food Sci 83:1063-1072.

250. Vivant A, Garmyn D, Maron P, Nowak V, Piveteau P. 2013. Microbial diversity and structure are drivers of the biological barrier effect against *Listeria monocytogenes* in soil. PLoS One 8:e76991.

251. Wan MLY, Forsythe SJ, El-Nezami H. 2019. Probiotics interaction with foodborne pathogens: a potential alternative to antibiotics and future challenges. Crit Rev Food Sci Nutr 59:3320-3333.

252. Wang C, Dong D, Strong P, Zhu W, Ma Z, Qin Y, Wu W. 2017. Microbial phylogeny determines transcriptional response of resistome to dynamic composting processes. Microbiome 5:1-15.

253. Wang D, Huber A, Dunfield K, Murray K, Wu F, Warriner K. 2018. Comparative persistence of *Salmonella* and *Escherichia coli* O157: H7 in loam or sandy loam soil amended with bovine or swine manure. Can J Microbiol 64:979-991.

254. Watabe M, Rao J, Stewart T, Xu J, Millar B, Xiao L, Lowery C, Dooley J, Moore J. 2003. Prevalence of bacterial faecal pathogens in separated and unseparated stored pig slurry. Lett Appl Microbiol 36:208-212.

255. Weller D, Wiedmann M, Strawn LK. 2015. Irrigation is significantly associated with an increased prevalence of *Listeria monocytogenes* in produce production environments in New York State. J Food Prot 78:1132-1141.

256. Wilkinson K, Tee E, Tomkins R, Hepworth G, Premier R. 2011. Effect of heating and aging of poultry litter on the persistence of enteric bacteria. Poult Sci 90:10-18.

257. Xiao Y, Zeng G, Yang Z, Ma Y, Huang C, Xu Z, Huang J, Fan C. 2011. Changes in the actinomycetal communities during continuous thermophilic composting as revealed by denaturing gradient gel electrophoresis and quantitative PCR. Bioresour Technol 102:1383-1388.

258. Xue C, Penton CR, Shen Z, Zhang R, Huang Q, Li R, Ruan Y, Shen Q. 2015. Manipulating the banana rhizosphere microbiome for biological control of Panama disease. Sci. Rep. 5:11124.

259. You Y, Rankin SC, Aceto HW, Benson CE, Toth JD, Dou Z. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. Appl Environ Microbiol 72:5777-5783.

260. Zeller W. 2006. Status of biocontrol methods against fire blight. Phytopathol.Pol 39:71-78.

261. Zhao T, Doyle MP, Zhao P. 2004. Control of *Listeria monocytogenes* in a biofilm by competitive-exclusion microorganisms. Appl Environ Microbiol 70:3996-4003. doi:10.1128/AEM.70.7.3996-4003.2004 [doi].

262. Zhao T, Podtburg TC, Zhao P, Chen D, Baker DA, Cords B, Doyle MP. 2013. Reduction by competitive bacteria of *Listeria monocytogenes* in biofilms and *Listeria bacteria* in floor drains in a ready-to-eat poultry processing plant. J Food Prot 76:601-607.

263. Zhao T, Podtburg TC, Zhao P, Schmidt BE, Baker DA, Cords B, Doyle MP. 2006. Control of Listeria spp. by competitive-exclusion bacteria in floor drains of a poultry processing plant. Appl Environ Microbiol 72:3314-3320. doi:72/5/3314 [pii].

264. Zhao X, Zhong J, Wei C, Lin C, Ding T. 2017. Current perspectives on viable but non-culturable state in foodborne pathogens. Front. Microbiol. 8:580.

265. Zhong X, Ma S, Wang S, Wang T, Sun Z, Tang Y, Deng Y, Kida K. 2018. A comparative study of composting the solid fraction of dairy manure with or without bulking material: performance and microbial community dynamics. Bioresour Technol 247:443-452.

266. Zhou Z, Zuber S, Campagnoli M, Moser M, Devlieghere F, Uyttendaele M. 2020. Decontamination effect of hot-air drying against bacterial pathogen and surrogate strains on basil leaves, from laboratory to pilot scale settings. LWT 122:109035.

267. Zhou Z, Zuber S, Campagnoli M, Putallaz T, Devlieghere F, Uyttendaele M. 2019. Effect of mild steaming treatment on the inactivation of *Salmonella*, *Listeria monocytogenes*, *Escherichia coli* O157: H7 and their surrogates on black peppercorns. Food Control 106:106726.

268. Zhu Q, Gooneratne R, Hussain MA. 2017. *Listeria monocytogenes* in fresh produce: outbreaks, prevalence and contamination levels. Foods 6:21.

CHAPTER TWO

TESTING A NON-PATHOGENIC SURROGATE MICROORGANISM FOR VALIDATING DESICCATION-ADAPTED *SALMONELLA* INACTIVATION IN PHYSICALLY HEAT-TREATED BROILER LITTER

ABSTRACT

Thermal resistance of desiccation-adapted *Salmonella* Senftenberg 775/W was compared with that of *Enterococcus faecium* NRRL B-2354 in aged broiler litter. Aged broiler litter with 20, 30, and 40% moisture contents were inoculated separately with desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 at ca. 5~6 log CFU/g, and then heat-treated at 75, 85, and 150°C. At all tested temperatures, desiccation-adapted *E. faecium* NRRL B-2354 was more heat-resistant than desiccation-adapted *S.* Senftenberg 775/W ($P<0.05$). During the treatments at 75 and 85°C, *E. faecium* NRRL B-2354 in aged broiler litter with all moisture contents was reduced by 2.9- to 4.1- log, and was above the detection limit of direct plating (1.3 log CFU/g), whereas *S.* Senftenberg 775/W could not be detected by enrichment (> 5- log reductions) during holding time at these temperatures. At 150°C, *E. faecium* NRRL B-2354 in aged broiler litter with 20 and 30% moisture contents was still detectable by enrichment after heat exposure for up to 15 min, whereas *S.* Senftenberg 775/W in aged broiler litter with all moisture contents could not be detected throughout the entire treatment. Our results revealed that *E. faecium* NRRL B-2354 can be used as a surrogate for *Salmonella* to validate the thermal processing of poultry litter by providing a sufficient safety margin. This study provides a practical tool for poultry litter processors to evaluate the effectiveness of their thermal processing.

INTRODUCTION

Poultry litter is a mixture of poultry excreta, feathers, spilled feed, and bedding materials. Approximately 10.2 million tons of poultry litter are generated annually on U.S. poultry farms (*13*). Since poultry litter contains nitrogen, phosphorus, and potassium which can be used as nutrients for plants, it has great value to serve as a biological soil amendment for crop production (*24*). However, poultry litter may harbor a variety of human pathogens that can contaminate fresh produce and cause foodborne illnesses (*7*). *Salmonella* spp., an important foodborne pathogen, is the most frequently isolated pathogen from poultry litter (*21*). Therefore, direct application of raw poultry litter to agricultural land for growing fresh produce should be avoided or closely monitored.

As recommended by the U.S. Food Safety and Modernization Act (FSMA) Produce Safety Rule, animal manure can be physically heat-treated to create a dried, pelleted material that is low in microbial populations (*25*). Physical heat treatment after or without composting has been used to reduce or kill pathogens in poultry litter (*8*, *9*, *15*). However, some pathogenic bacterial cells may become acclimatized to desiccation under the dry environment in poultry litter. The induced desiccation stress response makes those bacterial cells more heat-resistant to the subsequent high temperature (*5*). If the heat-resistant, desiccation-adapted *Salmonella* cells survive thermal processing of poultry litter, or the heat treatment is not uniform, use of this heat-treated litter as fertilizer on food crops can pose a potential threat to fresh produce safety. Therefore, it is warranted to evaluate the effectiveness of different treatment conditions during commercial thermal processing of poultry litter.

Due to biosafety concerns, pathogens cannot be tested directly in commercial manure processing environments. Hence, a non-pathogenic surrogate microorganism that behaves similar to the pathogen when exposed to the same treatment conditions is needed (*19*). In order to conduct any challenge studies in industrial settings, a lab-scale validation study comparing the surrogate and the pathogen under the same conditions should be performed (*22)*.

The effectiveness of using avirulent *Salmonella*, *Escherichia coli*, or enterococci as surrogates for *Salmonella* during food processing, produce contamination, and animal waste- related research has been extensively evaluated (*2*, *18*). For example, *Enterococcus faecium* NRRL B-2354, a widely used surrogate microorganism for human pathogens, is genetically and phenotypically distinct from clinical strains of *E. faecium*, and lacks functional copies of enterococcal virulence genes (*16*). *E. faecium* NRRL B-2354 has been used in thermal process validation of various food products, such as almonds, pistachios, and cereals (*12*). Previous research also demonstrated that indigenous enterococci can be used to validate the thermal processing of poultry litter, as it can predict the survival behaviors of *Salmonella* under various heat treatment conditions (*6*). Nonetheless, one limitation of using indigenous enterococci as indicator microorganisms is the uncertainty of species and population level of enterococci in each batch of poultry litter. Currently, there are no available published studies on the use of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* during thermal processing of animal waste products. Therefore, the objective of this study was to evaluate *E. faecium* NRRL

B-2354 as a surrogate for desiccation-adapted *Salmonella* inactivation in physically heat-treated poultry litter.

MATERIALS AND METHODS

Sample preparation. Aged broiler litter was collected from Cobb broiler chickens (Organic Farms, Livingston, CA). Litter from chicken houses was removed annually, followed by partial windrow composting for 7~10 days. After composting, the litter was then screened to remove rice hulls. Broiler litter samples used for the following experiments were dried under a chemical fume hood until moisture content was reduced to < 20%, and then screened to a particle size of < 3 mm using a sieve (sieve pore size, 3 by 3 mm) to reduce the sample heterogeneity. Sufficient samples were prepared for the entire study and stored in a sealed container at 4°C until use. Moisture content was measured with a moisture analyzer (Model IR-35, Denver Instrument, Denver, CO).

Bacterial strains and culture conditions. *S. enterica* Senftenberg ATCC 43845 (775/W), as *S.* Senftenberg 775/W, was identified as the most heat resistant among four *Salmonella* serotypes tested during thermal processing of aged broiler litter in a previous study (*5*). *E. faecium* NRRL B2354 (ATCC 8459) was evaluated as a potential *Salmonella* surrogate for thermal process validation of broiler litter. Both *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 were induced to rifampin resistance (100 µg/ml) using the gradient plate method (*23*), the rifampin resistant mutant was selected for each strain. Each strain was grown overnight at 37°C in tryptic soy broth (TSB; Becton, Dickinson and company, Sparks, MD) containing 100 µg rifampin/ml (TSB-R).

The overnight cultures were then washed three times with sterile 0.85% saline. For *S.* Senftenberg 775/W, the final pelleted cells were resuspended in 0.85% saline to the desired cell concentration (ca. 10^9 CFU/ml) by adjusting the optical density at 600 nm to ca. 0.7. Afterwards, the re-suspended *S.* Senftenberg 775/W culture was further concentrated 100 times (ca. 10^{11} CFU/ml) by centrifugation. As for *E. faecium* NRRL B-2354, the desired cell concentration was ca. 10^8 CFU/ml (OD_{600}=0.5). Enumeration of *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 was performed on Xylose-Lysine-Tergitol 4 agar (XLT-4; Becton, Dickinson and company) and enterococcosel agar (EA; Becton, Dickinson and company), respectively, and those two agars were supplemented with 100 μg rifampin/ml.

Desiccation adaptation. The aged broiler litter used for desiccation adaptation was first exposed to greenhouse conditions for 15 days to lower the ammonia contents, in order to minimize the population reduction during desiccation adaptation (*8*). The broiler litter was then adjusted to the desired moisture contents of 20, 30, and 40% with sterile tap water. The washed *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 cells were added (1:10, v/w) separately into 100 g of broiler litter with reduced ammonia content, mixed well using a sterile blender (Professional 600 Series, KitchenAid, Inc., St. Joseph, MI) at a final concentration of ca. 10^{10} and 10^7 CFU/g, respectively, and then held in a sterile tray (8.8 × 6.3 × 6.1 inches) covered loosely by sterile aluminum foil. After 24-h desiccation adaptation at room temperature, the inoculated aged broiler litter was further mixed (1:100, w/w) with 500 g of regular broiler litter with the same moisture content using the sterile blender. This thoroughly blended sample was used for subsequent heat

treatment. The initial populations of *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in broiler litter were enumerated immediately before thermal treatments.

Thermal inactivation. Twenty-gram samples in duplicate were distributed evenly inside sterile aluminum pans (I.D. 10 cm). A temperature-controlled convectional oven (Binder Inc., Bohemia, NY) was initially set 5°C higher than the target temperature. Aluminum pans with the inoculated samples were quickly placed at two different locations on the oven shelf, and then exposed to 75, 85, and 150°C, separately. Temperature was monitored using T-type thermocouples (DCC Corp., Pennsauken, NJ), with one thermocouple monitoring the oven chamber temperature and the others being inserted into litter samples at two different locations. When the interior temperature of the litter samples reached the target temperature (0 h), the oven temperature setting was readjusted to the target temperature. Broiler litter samples in duplicate were withdrawn quickly from the oven at 0 h and every 0.5 h during the holding time up to 3 h for determination of microbial populations. For heat treatments at 75 and 85°C, broiler litter samples were also collected every 0.5 h during the come-up times. Samples were transferred into a Whirl-Pak bag (Nasco, Fort Atkinson, WI), and placed immediately in an ice-water bath to cool the samples and minimize further cell death. For heat treatment at 150°C, duplicate samples were withdrawn every 15 min up to 60 min and enriched directly to test if *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 survived the treatment.

Bacterial enumeration. The surviving populations of *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 were enumerated, using a modified two-step overlay method to

allow heat-injured cells to resuscitate (*5*). Both XLT-4 and EA were supplemented with 100 µg rifampin/ml as the selective media, and tryptic soy agar (TSA; Becton, Dickinson and company) was used as the nonselective media. Samples which were negative for *S.* Senftenberg 775/W by direct plating method were pre-enriched in universal pre-enrichment broth (UPB; Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV; Becton, Dickinson and company) supplemented with 100 µg rifampin/ml. After 24-h incubation at 42°C, enriched cultures were then plated onto XLT-4 supplemented with 100 µg rifampin/ml. Samples negative for *E. faecium* NRRL B-2354 by the direct plating method were pre-enriched in UPB supplemented with 100 µg rifampin/ml. After 24-h incubation at 37°C, enriched cultures were then plated onto EA supplemented with 100 µg rifampin/ml.

Thermal inactivation kinetics. Mathematical models, including Weibull, modified Gompertz, and log-logistic models, were applied to fit the thermal inactivation curves (*10*). For all the following models, N_0 = initial population of bacterial cells (CFU/g in dry weight); N= population of survivors after a treatment time t (CFU/g in dry weight). An adjusted regression coefficient R^2 was used to evaluate and compare the goodness-of-fit of the proposed models.

The Weibull model describes the phenomenon that bacterial cells in a population experience different resistances (*20*):

$$\log\frac{N}{No} = -bt^n$$

where *b* and *n* are scale and shape factors, respectively.

The modified Gompertz model was designed primarily to model the asymmetrical sigmoidal shape of a growth curve and was later developed to fit inactivation kinetics (*14*):

$$\log\frac{N}{No} = Ce^{-e^{BM}} - Ce^{-e^{-B(t-M)}}$$

where M = time (min) at which the absolute death rate is maximum; B = relative death rate (CFU/g per min) at M; C = difference in value of the upper and lower asymptotes (CFU/g).

The log-logistic model was proposed to describe the non-linear thermal inactivation of microorganisms (*11*):

$$\log\frac{N}{No} = \frac{A}{1+e^{4\sigma(\tau-logt)/A}} - \frac{A}{1+e^{4\sigma(\tau-logt0)/A}}$$

Where A= difference in value of the lower and upper asymptotes; σ = maximum rate (log CFU/g) of inactivation; τ = log time (log min) to the maximum rate of inactivation.

Statistical analysis. All experiments were conducted in two separate trials. Plate count data were converted to log CFU/g in dry weight. Differences among treatments were analyzed by Analysis of Variance (ANOVA) followed by least significant differences (LSD) and were considered to be significant when $P < 0.05$. SigmaPlot 12.3 (Systat Software Inc., San Jose, CA) was used for data analysis and curve fitting.

RESULTS

The come-up times for heating aged broiler litter with different moisture contents at 75 and 85°C ranged from 90 to 150 min (data not shown). After come-up time at 75 and 85°C, the populations of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter decreased in all samples. Specifically, at 75°C, after come-up time, ca. 4- to 5.5-log of *Salmonella* were inactivated, whereas reductions of *E. faecium* NRRL B-2354 were 2.5, 2.7, and 3.0 log CFU/g for 20, 30, and 40% moisture contents, respectively (Figure 2.1). After come-up time at 85°C, regardless the moisture contents, more than 5- log reductions of *S.* Senftenberg 775/W was observed as compared with 2.8-, 3.8-, and 3.5- log reductions in the *E. faecium* NRRL B-2354 population for 20, 30, and 40% moisture contents, respectively (Figure 2.2).

Both *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 were inactivated much faster when temperature and moisture content of aged broiler litter were increased (Figures 2.1 and 2.2). For example, for the entire heat treatment including come-up and holing times, the log reductions of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 30% moisture content were 2.9 and 4.0 log CFU/g at 75 and 85°C (Figures 2.1B and 2.2B), respectively, as compared to 3.4 and 4.1 log CFU/g in broiler litter with 40% moisture content, respectively (Figures 2.1C and 2.2C). At 75°C, desiccation-adapted *S.* Senftenberg 775/W in aged broiler litter with 20% moisture content (Figures 2.1A) was detectable by enrichment during come-up time (90 min), whereas it could not be detected after 60 min in aged broiler litter with 40% moisture content (Figures 2.1C). After come-up time, the population of *E. faecium* NRRL B-2354

stabilized around 1.5 to 2.5 log CFU/g at both 75 and 85°C, indicating no further reduction for all treatments during the rest of heat treatment.

As shown in Figure 2.3, *E. faecium* NRRL B-2354 yielded a significantly ($P<0.05$) lower average log reduction (24 of the 24 data points) as compared with *S.* Senftenberg 775/W, indicating a conservative response for *E. faecium* NRRL B-2354 during heat treatment. For the linear regression results between the mean log reductions of *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in broiler litter with various moisture contents at 75°C (data not shown), the log reductions for *E. faecium* NRRL B-2354 were below those for *S.* Senftenberg 775/W. By summarizing all thermal inactivation data under different combinations of temperature and moisture, our results clearly demonstrated that a >1.2 ~ 2.7- log reductions of *E. faecium* NRRL B-2354 can predict >5-log reductions of *Salmonella* depending on heating temperature and poultry litter moisture combinations (Table 2.1).

At 150°C, desiccation-adapted *E. faecium* NRRL B-2354 also displayed extended survival compared to desiccation-adapted *S.* Senftenberg 775/W. Desiccation-adapted *S.* Senftenberg 775/W in aged broiler litter with all moisture levels could not be detected throughout the entire treatment. However, desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20 and 30% moisture contents was still detectable by enrichment for up to 15 min heat treatment, while the same culture in aged broiler litter at 40% moisture content could not be detected throughout the entire treatment (60 min) (data not shown).

Due to the lack of sufficient data points for modeling, inactivation curves of desiccation-adapted *S.* Senftenberg 775/W were not fitted into mathematical models. The Weibull, modified Gompertz, and log-logistic models were fitted into the survival curves of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% moisture contents at 75 and 85°C. For fitting the inactivation data of desiccation-adapted *E. faecium* NRRL B-2354, the modified Gompertz model produced the best description, with mean adjusted R^2 values of 0.97 and 0.99 at 75 and 85°C, respectively (Table 2.2). The "*B*" values (relative death rate in CFU/g per min) in the Gompertz model were lower at 75°C than at 85°C in aged broiler litter with the same moisture content, which suggested a slower population decrease at a lower temperature (Table 2.3). The "*C*" value (difference in value of the upper and lower asymptopes in CFU/g) became higher as moisture content increased from 20 to 40%. This indicated a higher population reduction at a higher moisture content, except at 75°C where no difference was found for "*C*" values between broiler litter with 20 and 30% moisture contents. The "*M*" value (time at which the absolute death rate is maximum) obtained from the Modified Gompertz model displayed no dependencies on either temperature or moisture content, and it is therefore difficult to draw a definite conclusion from this parameter.

DISCUSSION

In the present study, microbial populations in aged broiler litter decreased during exposure to tested temperatures, with shorter survival at 85°C than at 75°C. Our data also clearly revealed that bacterial cells became less heat-resistant to heat when the moisture

content of aged broiler litter was increased from 20 to 40%, which are in agreement with previously published data of the thermal inactivation of *S. enterica* in poultry litter (*5*, *6*, *8*, 9). Modeling of the survival data of desiccation-adapted *E. faecium* NRRL B-2354 using the modified Gompertz model also supported above findings.

S. Senftenberg 775/W has been used extensively for thermal validation studies as this *Salmonella* strain has high heat tolerance as reported in previous studies. In considering that the thermal processing should be adequate to kill the most heat-resistant harmful microorganism that may occur in ground beef, Ma et al. (*17*) used *S.* Senftenberg 775/W for an in-plant critical control point validation study on ground beef. Cuervo et al. (*12*) investigated the thermal resistance of *S.* Senftenberg 775/W during blanching with the organism inoculated onto almond surfaces. Their results suggested that the almond should be blanched at 88°C for a minimum of 63 s to achieve a 4-log reductions in the *S.* Senftenberg 775/W population. Ceustermans et al. (*3*) also used *S.* Senftenberg 775/W to determine the hygienic safety of biowastes and garden wastes during composting. They concluded that *S.* Senftenberg 775/W can be inactivated within 10 h of composting, when the temperature of the compost heap with 60~65% moisture contents was 60°C. However, as *S.* Senftenberg 775/W is not considered as biosafety level 1 (BSL-1), an appropriate, non-pathogenic surrogate microorganism is needed for validation studies.

Attenuated pathogens or non-pathogenic surrogate microorganisms have been used in a variety of validation studies in place of foodborne pathogens to avoid introducing pathogens into commercial processing environments (*22*). Ease of use is a necessary attribute where “ease” refers to the ability to produce the microorganism in

high populations, the simplicity in detection, and the lack of adverse effects on industrial processing environment and workers. Moreover, to be considered as appropriate surrogates for use in validation studies, the microorganisms must possess similar key characteristics to adequately represent the target pathogenic microorganism. Theoretically, surrogate microorganism(s) for validating thermal processing should be non-pathogenic microorganism(s) that provides a similar response as the target microorganism(s) when subjected to the same treatment conditions (*1*). To avoid false negative results, another critical criterion in selecting a surrogate microorganism is the confirmation that the surrogate is more heat-resistant than the pathogen of concern (*17*).

E. faecium NRRL B-2354, classified in the BSL-1 category, is genetically stable, can be easily cultivated and enumerated using standard microbiological methodology, does not form biofilms, and does not produce undesirable odor (*4*). Due to these properties, *E. faecium* NRRL B-2354 has been used as a surrogate for *Salmonella* in validating thermal processing in the food industry (*16*). A recent study by Villa-Rojas et al. (*26*) demonstrated that *E. faecium* NRRL B-2354 was a suitable surrogate for *S.* Enteritidis PT 30 during heat processing of organic wheat flour, with results indicating a 2-log greater reductions of *S.* Enteritidis PT 30 than *E. faecium* NRRL B-2354 when subjected to an equivalent treatment (75°C and $a_w = 0.25$). Ma et al. (*17*) reported *D*-values of *E. faecium* NRRL B-2354 in ground beef with 4 and 12% fat contents *at* 58~68°C were 4.4 to 17.7 and 3.6 to 14.6 times greater, respectively, than those of *S.* Senftenberg 775/W. They thus concluded that depending on the margin of safety desired,

processors could use *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* for validation studies of thermal processes.

In the present study, data indicated that *E. faecium* NRRL B-2354 had higher thermal resistance as compared to *S.* Senftenberg 775/W under the same treatment conditions. Due to the relatively longer come-up time to heat the complex compost matrix to the desired tested temperatures, it is difficult to compare D-values between these two microorganisms. However, the plate count data revealed that the heat treatment resulting in a 1~3-log reductions of *E. faecium* NRRL B-2354 can yield to a ca. 5.5- log reductions of *S.* Senftenberg 775/W (Table 2.1). Overall, the thermal inactivation data obtained in our study were consistent with the above published studies.

Our results demonstrated that desiccation-adapted *E. faecium* NRRL B-2354 was more heat-resistant at 75, 85, and 150°C as compared to desiccation-adapted *S.* Senftenberg 775/W in poultry litter. In conclusion, our findings indicated that *E. faecium* NRRL B-2354 can be used as a surrogate for *S. enterica* to provide a sufficient safety margin when validating the thermal processing of poultry litter. The thermal inactivation data obtained from this study may assist poultry litter processors in validating their processes to ensure the microbiological safety of physically heat-treated poultry litter. However, due to the controlled experimental conditions used in our study, the results could differ from those generated under the commercial plant conditions. Further study using *E. faecium* NRRL B-2354 as a surrogate in validating a commercial poultry litter heat processing plant is currently underway.

REFERENCES

1. Bianchini, A., J. Stratton, S. Weier, T. Hartter, B. Plattner, G. Rokey, G. Hertzel, L. Gompa, B. Martinez, and K.M. Eskridge. 2014. Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. *J. Food Prot.* 77: 75-82.

2. Borowski, A.G., S.C. Ingham, and B.H. Ingham. 2009. Validation of ground-and-formed beef jerky processes using commercial lactic acid bacteria starter cultures as pathogen surrogates. *J. Food Prot.* 72: 1234-1247.

3. Ceustermans, A., D. De Clercq, A. Aertsen, C. Michiels, J. Coosemans, and J. Ryckeboer. 2007. Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. *J. Appl Microbiol.* 103: 53-64.

4. Ceylan, E., and D. A. Bautista. 2015. Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. *J. Food Prot.* 78: 934-939.

5. Chen, Z., J. Diao, M. Dharmasena, C. Ionita, X. Jiang, and J. Rieck. 2013. Thermal inactivation of desiccation-adapted *Salmonella* spp. in aged chicken litter. *Appl Environ Microbiol.* 79: 7013-7020.

6. Chen, Z., and X. Jiang. 2017. Selection of indigenous indicator microorganisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter. *J. Appl Microbiol.* 122: 1558-1569.

7. Chen, Z., and X. Jiang. 2014. Microbiological safety of chicken litter or chicken litter-based organic fertilizer: A review. *Agric.* 4: 1-29.

8. Chen, Z., H. Wang, C. Ionita, F. Luo, and X. Jiang. 2015. Effect of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* spp. *Appl Environ Microbiol.* 81: 6883-6889.

9. Chen, Z., H. Wang, and X. Jiang. 2015. Developing a two-step heat treatment for inactivating desiccation-adapted *Salmonella* spp. in aged chicken litter. *Foodborne Pathog Dis.* 12: 104-109.

10. Chen, Z., and C. Zhu. 2011. Modelling inactivation by aqueous chlorine dioxide of *Dothiorella gregaria Sacc.* and *Fusarium tricinctum (Corda) Sacc.* spores inoculated on fresh chestnut kernel. *Lett. Appl Microbiol.* 52: 676-684.

11. Cole, M.B., K.W. Davies, G. Munro, C.D. Holyoak, and D.C. Kilsby. 1993. A vitalistic model to describe thermal inactivation of *Listeria monocytogenes*. *J. Ind Microbiol.* 12: 232–237.

12. Cuervo, M.P., L.M. Lucia, and A. Castillo. 2016. Efficacy of traditional almond decontamination treatments and electron beam irradiation against heat-resistant *Salmonella* strains. *J. Food Prot.* 79: 369-375.

13. Dunkley, C.S., D.L. Cunningham, and G.H. Harris. 2014. The value of poultry litter in South Georgia. UGA Cooperative Extension Bull. Available at: https://secure.caes.uga.edu/extension/publications/files/pdf/B%201386_3.PDF Accessed 15 March, 2018.

14. Gibson, A.M., N. Bratchell, and T.A. Roberts. 1988. Predicting microbial growth:

growth responses of *Salmonellae* in a laboratory medium as affected by pH, sodium chloride, and storage temperature. *Int J. Food Microbiol.* 6:155–178.

15. Kim, J., J. Diao, M.W. Shepherd Jr., R. Singh, S.D. Heringa, C. Gong, and X. Jiang. 2012. Validating thermal inactivation of *Salmonella* spp. in fresh and aged chicken litter. *Appl Environ Microbiol.* 78: 1302-1307.

16. Kopit, L. M., E. B.Kim, R. J. Siezen, L. J. Harris, and M. L. Marco. 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. *Appl Environ Microbiol.* 80: 1899-1909.

17. Ma, L., J. L. Kornacki, G. Zhang, C. M. Lin, and M. P. Doyle. 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. *J. Food Prot.* 70: 952-957.

18. Mak, P.P., B.H. Ingham, and S.C. Ingham. 2001. Validation of apple cider pasteurization treatments against *Escherichia coli* O157: H7, *Salmonella*, and *Listeria monocytogenes*. *J. Food Prot.* 64: 1679-1689.

19. Niebuhr, S. E., A. Laury, G. R. Acuff, and J. S. Dickson. 2008. Evaluation of nonpathogenic surrogate bacteria as process validation indicators for *Salmonella enterica* for selected antimicrobial treatments, cold storage, and fermentation in meat. *J. Food Prot.* 71: 714-718.

20. Peleg, M., and M.B. Cole. 1998. Reinterpretation of microbial inactivation curves. *Crit Rev Food Sci.* 38:353–380.

21. Poppe, C., R. J. Irwin, C. M. Forsberg, R. C. Clarke, and J. Oggel. 1991. The prevalence of *Salmonella enteritidis* and other *Salmonella* spp. among Canadian

registered commercial layer flocks. *Epidemiol Infect.* 106: 259-270.

22. Sinclair, R. G., J. B. Rose, S. A. Hashsham, C. P. Gerba, and C. N. Haas. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Appl Environ Microbiol*. 78: 1969-1977.

23. Smith, J.L., R.C. Benedict, and S.A. Palumbo. 1982. Protection against heat-injury in *Staphylococcus aureus* by solutes. *J. Food Prot.* 45: 54-58.

24. Tewolde, H., K. R. Sistani, and D. E. Rowe. 2005. Broiler litter as a sole nutrient source for cotton: Nitrogen, phosphorus, potassium, calcium, and magnesium concentrations in plant parts. *J. Plant Nutr.* 28: 605-619.

25. U.S. Food and Drug Administration (USFDA). 2015. Standards for the growing, harvesting, packing, and holding of produce for human consumption. FDA Food Safety Modernization Act (FSMA). USFDA, Silver Spring, MD. Available at: https://www.fda.gov/downloads/food/guidanceregulation/fsma/ucm470746.pdf Accessed 15 March, 2018.

26. Villa-Rojas, R., M. J. Zhu, B.P. Marks, and J. Tang. 2017. Radiofrequency inactivation of *Salmonella* Enteritidis PT 30 and *Enterococcus faecium* in wheat flour at different water activities. *Biosyst. Eng.* 156: 7-16.

Table 2.1 Relationships between *Salmonella* and surrogate reductions during heat treatment at 75 and 85ºC in aged broiler litter [a]

Temperature (°C)	Moisture content (%)	*Salmonella* reduction (log CFU/g)	*E. faecium* NRRL B-2354 reduction (log CFU/g)
75	20	5.55±0.12a	2.59±0.28a
	30	5.27±0.65a	2.52±0.55ab
	40	5.44±0.20a	1.95±0.34abc
85	20	5.62±0.10a	1.23±0.72c
	30	5.79±0.00a	1.27±0.55bc
	40	5.71±0.43a	1.18±0.52c

[a] Data were expressed as means ± standard deviations. Levels not connected by the same lowercase letter within the column are significant different ($P < 0.05$).

Table 2.2 Goodness-of-fit of the Weibull, modified Gompertz, and log-logistic models for the survival curves of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% moisture contents at 75 and 85°C

Temperature (°C)	Moisture content (%)	Adjusted R^2 with the following models		
		Weibull	Modified Gompertz	Log-logistic
75	20	0.911	0.992	0.991
	30	0.911	0.964	0.960
	40	0.924	0.954	0.948
85	20	0.931	0.983	0.872
	30	0.870	0.997	0.998
	40	0.928	0.992	0.987

Table 2.3 Parameters of the Weibull, modified Gompertz, and log-logistic models for the survival curves of desiccation-adapted *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, and 40% moisture contents at 75 and 85°C

Temperature (°C)	Moisture content (%)	Parameters associated with following models		
		Weibull	Modified Gompertz	Log-logistic
75	20	b=0.501, n=0.326	M=28.358, B=0.035, C=3.009	A=2.887, σ=-3.739, τ=1.615
	30	b=0.246, n=0.439	M=39.471, B=0.024, C=3.005	A=2.947, σ=-3.245, τ=1.792
	40	b=0.333, n=0.396	M=-27.8891, B=0.015, C=6.375	A=3.404, σ=-2.768, τ=1.809
85	20	b=0.646, n=0.299	M=23.127, B=0.037, C=3.465	A=6.955, σ=-1.453, τ=2.672
	30	b=0.929, n=0.275	M=32.035, B=0.054, C=3.977	A=4.009, σ=-7.044, τ=1.584
	40	b=0.479, n=0.385	M=26.223, B=0.021, C=4.954	A=4.337, σ=-4.352, τ=1.773

Figure 2.1 Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20 (A), 30 (B), and 40% (C) moisture contents at 75°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.30 log CFU/g).

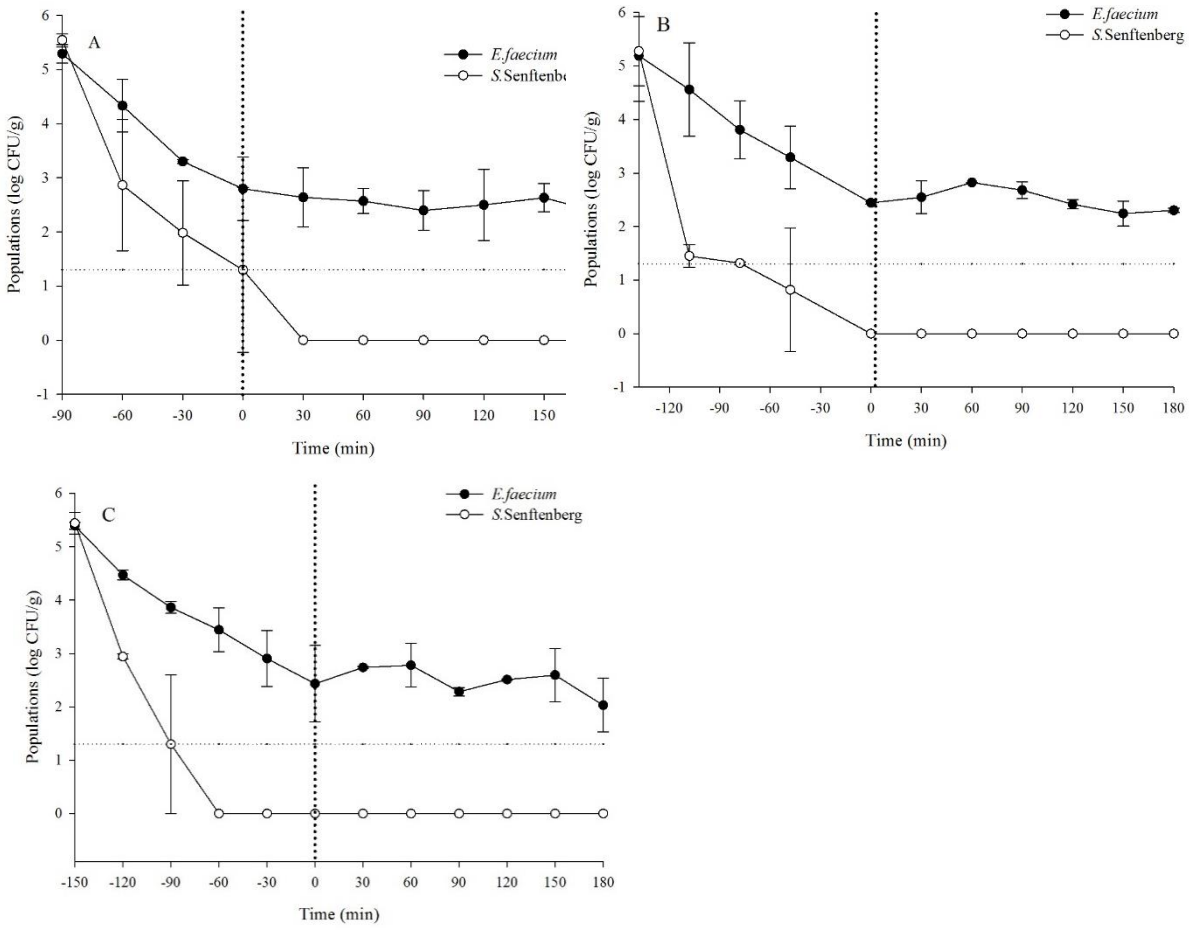


Figure 2.2 Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20 (A), 30 (B), and 40% (C) moisture contents at 85°C. Inactivation curves during come-up times (on the left of the vertical dotted line) and during holding times (on the right of the vertical dotted line) are shown. The horizontal dotted line indicates that Salmonella was detectable only by enrichment (detection limit by direct plating: 1.3 log CFU/g).

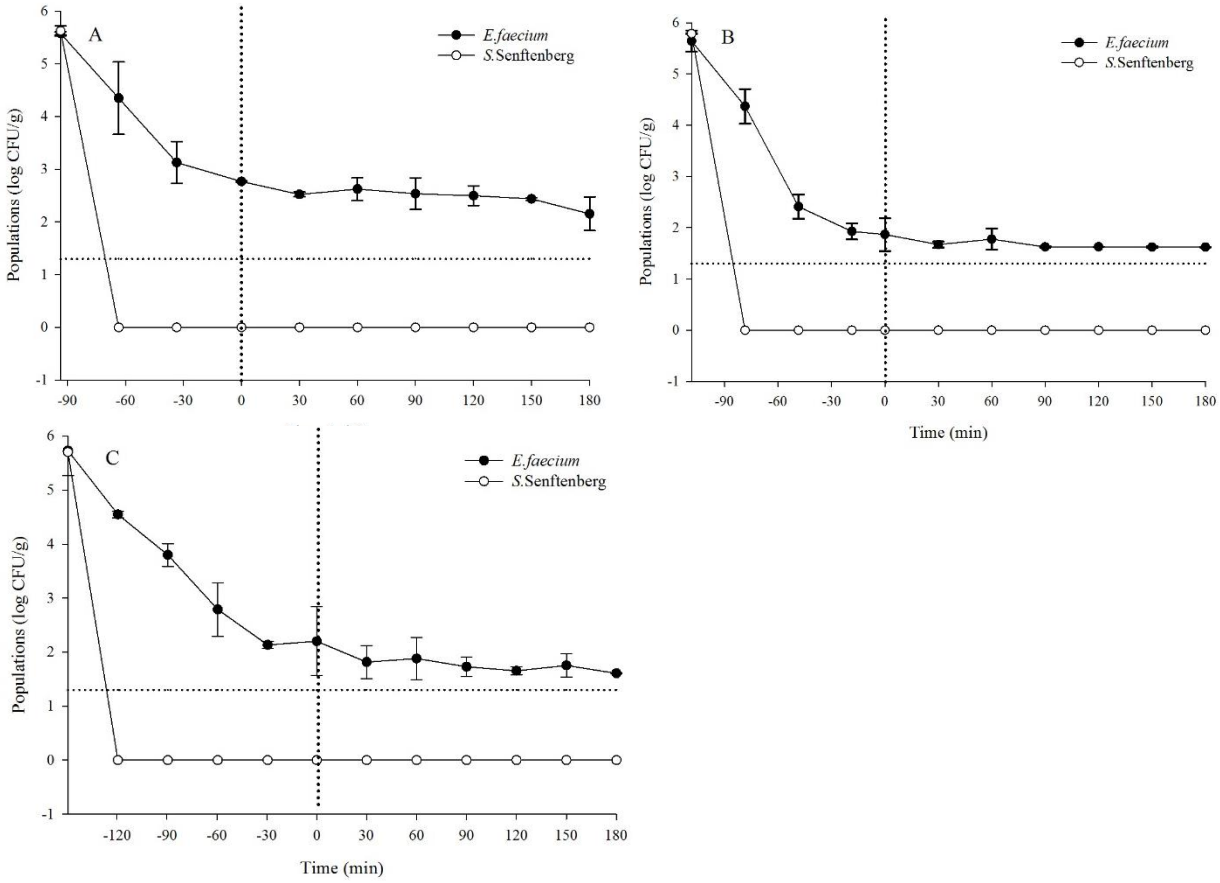
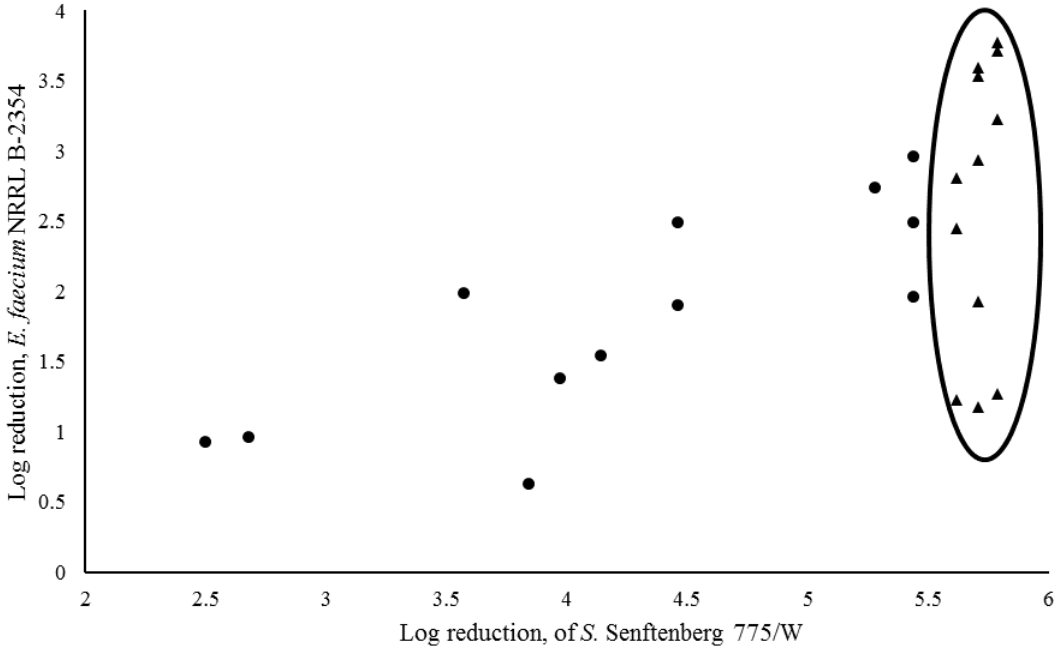


Figure 2.3 Direct comparison between the mean log reductions for desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in aged broiler litter with 20, 30, 40% moisture contents at 75°C (solid circle data points outside the circle) and 85°C (solid triangle data points inside the circle) (n=24). Each treatment was conducted with two separate trials.



CHAPTER THREE

PLANT-SCALE VALIDATION OF PHYSICAL HEAT TREATMENT OF POULTRY LITTER COMPOSTS USING SURROGATE AND INDICATOR MICROORGANISMS FOR *SALMONELLA*

ABSTRACT

This study selected and used indicator and surrogate microorganisms for *Salmonella* to validate the processes for physically heat-treated poultry litter compost in litter processing plants. Initially laboratory validation studies indicated that 1.2- to 2.7-log or more reductions of desiccation-adapted *Enterococcus faecium* NRRL B-2354 were equivalent to > 5-log reductions of desiccation-adapted *Salmonella* Senftenberg 775/W in poultry litter compost, depending on treatment conditions and compost types. Plant validation studies were performed in one turkey litter compost processor and one laying hen litter compost processor. *E. faecium* was inoculated at ca.7 log CFU g^{-1} into the turkey litter compost and at ca. 5 log CFU g^{-1} into laying hen litter compost with respectively targeted moisture contents. The thermal processes in the two plants yielded 2.8 - > 6.4 log CFU g^{-1} (> 99.86%) reductions *E. faecium* of the inoculated. Similarly, for the processing control samples, reductions of presumptive indigenous enterococci were in the order of 1.8-3.7 log CFU g^{-1} (98.22 % to 99.98 %) of the total naturally present. In contrast, there were less reductions of indigenous mesophiles (1.7-2.9 log CFU) and thermophiles (0.4-3.2 log CFU g^{-1}). More indigenous enterococci were inactivated in the presence of higher moisture in the poultry litter compost. Based on the data collected under the laboratory conditions, the processing conditions in both plants were adequate to

reduce any potential *Salmonella* contamination of processed poultry litter compost by at least 5-log, even though the processing conditions varied among trials and plants.

INTRODUCTION

Poultry litter contains essential nutrients for crop growth (1) and is often used in organic farming as biological soil amendments of animal origin (BSAAO). Based on the Organic Materials Review Institute (OMRI)/USDA National Organic Program (NOP) and California Leafy Green Marketing Agreement rules, the use of raw manure on fresh produce for human consumption is discouraged due to the possible presence of human pathogens such as *Salmonella* spp. (2, 3). Either composting or physical heat is used to reduce pathogens in poultry litter used as organic fertilizer for growing fresh produce (4). According to the FDA Food Safety Modernization Act (FSMA) produce safety rule (5), the thermal process for animal manure or other biological soil amendments should be scientifically validated to satisfy the microbial standard requirement for *Salmonella* species, i.e. < 3 MPN per 4 grams. Unfortunately, there is very limited research on the microbiological safety of physically heat-treated poultry litter or poultry litter compost. Therefore, scientific data and practical validation methods for thermal inactivation of *Salmonella* are urgently needed for the industry.

The introduction of pathogenic strains of microorganisms into the industrial environment is not recommended for plant validation studies. Hence, indigenous or non-pathogenic bacteria with similar growth/survival characteristics to pathogens should be used as indicator or surrogate microorganisms to understand the growth/survival behaviors of

pathogens in industrial environments (6). Côté et al. (7) reported that a reduction of 1.6-4.2 log CFU ml^{-1} total coliforms and a reduction of 2.5-4.2 log CFU ml^{-1} indigenous *Escherichia coli* resulted in undetectable levels of indigenous *Salmonella* in swine slurries during anaerobic digestion. Besides, Qi et al. (8) also reported that a reduction of 91.1% enterococci and a reduction of 99.7% indigenous *E. coli* resulted in 99.3% of indigenous *Salmonella* in dairy manure during thermal anaerobic digestion. During composting or stockpiling, the counts of presumptive indigenous enterococci and mesophiles in poultry litter ranged from 3.0-7.5 log and 6.6-8.9 log CFU g^{-1}, respectively, which can provide sufficient populations for predicting the survival behaviors of *Salmonella* in poultry litter during subsequent thermal treatment (9, 10, 11). Likewise, our previous study (12) reported a correlation ($R^2 > 0.88$) between the mean log reductions of *Salmonella* Senftenberg 775/W with those of presumptive indigenous enterococci in turkey litter compost samples with 20-50% moisture contents at 75ºC as compared to total aerobic bacteria. This previous work indicated that presumptive indigenous enterococci could be an indicator for validating the effectiveness of thermal processing. Several bacterial species have been recommended as *Salmonella* surrogates in different matrices, such as *Pediococcus acidilactici* ATCC 8042 and *Enterococcus faecium* NRRL B-2354 in dry pet food (13), *E. faecium* NRRL B-2354 and *Pantoea agglomerans* SPS2F1 in almonds (14), and *Pediococcus* spp. in beef or turkey jerky (15, 16). Among those surrogates, *E. faecium* NRRL B-2354 has been widely used as a surrogate for *S. enterica* for validating the thermal processing of nuts and carbohydrate-protein meal (17, 18). The safety of *E. faecium* NRRL B-2354 as a surrogate has been

thoroughly studied by Kopit et al. (19) by sequencing, confirming that this strain lacks of genomic and phenotypic characteristics linked to nosocomial infections.

Our previous laboratory-based studies on the thermal inactivation of *Salmonella* in poultry litter compost have revealed that *Salmonella* cells were killed at 70 - 150ºC within 30 min to 6 h, depending on the bacterial physiological status (desiccation-adapted or not), moisture level, experimental design, and heat sources (moist heat or dry heat) (4, 10). Although these laboratory-based studies indicated that the thermal treatment conditions completely inactivated *Salmonella*, the conclusions could differ from those in the industrial settings, in which the processing capacity is scaled-up, and heterogeneity of poultry litter compost or treatments may exist. During a processing plant validation study, the effects of other operational factors, such as dryer construction and temperature, residence time, and characteristics of processing products, on microbial inactivation should also be evaluated (20). To date, there is no published study on validating the thermal processing of BSAAO in thermal processing plant settings.

The objectives of this study were to i) compare the thermal resistance of *S.* Senftenberg 775/W and its surrogate *E. faecium* NRRL B-2354 in poultry litter compost under laboratory conditions; ii) validate the thermal processing conditions for poultry litter compost in two litter compost processing plants using *Salmonella* surrogate and indicator microorganisms; and iii) determine the factors affecting the thermal inactivation of *Salmonella* surrogate and indicator microorganisms in industry settings.

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* Senftenberg 775/W (ATCC 43845) was used as a reference strain in the laboratory validation study, as it was identified as the most heat-resistant among four *Salmonella* strains tested during thermal processing of aged broiler litter in our previous laboratory-scale study (4). *E. faecium* NRRL B-2354 (ATCC 8459) was evaluated as a potential *Salmonella* surrogate for both laboratory and plant validation studies. Both strains were induced to rifampin resistance (100 μg ml^{-1}) using the gradient plate method (21). Each strain was grown overnight at 37°C in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) containing 100 μg rifampin ml^{-1} (TSB-R). The overnight cultures were then washed three times and resuspended with sterile 0.85% saline to the desired cell concentrations by measuring the optical density at 600 nm. *S.* Senftenberg 775/W and *E. faecium* were enumerated using Xylose-Lysine-Tergitol-4 agar (XLT-4; Becton, Dickinson and company, Sparks, MD) and enterococcosel agar (EA; Becton, Dickinson and Company, Sparks, MD), respectively, supplemented with 100 μg rifampin ml^{-1} (XLT-4-R and EA-R).

Selection of the recovery media for heat-injured *E. faecium* and presumptive indigenous enterococci using turkey litter compost. The turkey litter compost adjusted with 30% moisture content (a_w = 0.925) was inoculated with overnight grown *E. faecium* cells at a ratio of 1:100. The temperature of a controlled convection oven (Binder Inc., Bohemia, NY) was initially set at 80ºC. When the oven temperature reached 80ºC, sealed-Tyvek pouches (12.7 × 12.7 cm) containing 50 g turkey litter compost samples with or without *E. faecium* were placed on the oven shelf at the middle location, and then

exposed to the heat treatment for 60 min. The temperature was monitored constantly using T type thermocouples (DCC Corporation, Pennsauken, NJ), with one cord kept inside the oven chamber and other cords kept into the compost samples. When the interior of the compost reached the desired temperature (75ºC), the temperature setting of the oven was readjusted to maintain at this designated temperature. Samples were taken out at 30 and 60 min of heat exposure and were transferred into a sterile Whirl-Pak™ bag (700 ml; Nasco, Inc., Madison, WI) and placed immediately in an ice water bath to stop further cell death. Samples were then serially diluted with 0.85% saline and plated in duplicate on the following plates to evaluate the recovery efficiency with these media.

Tryptic soy agar (TSA; Becton, Dickinson and Company) was used as a nonselective medium, whereas EA and bile esculin azide agar (BEA; Becton, Dickinson and Company) were used as selective media for enterococci. EA and BEA supplemented with rifampin (100 μg ml^{-1}) were used for *E. faecium*. EA or BEA, EA or BEA alone, modified two-step overlay (OV) method with heat-injured cells were plated directly onto TSA and modified thin agar layer (TAL) method were compared for the recovery of heat-injured presumptive indigenous enterococci cells and *E. faecium*. For OV methods, after incubation at 37°C for 2 h to allow the recovery of injured cells, 7 ml of BEA or EA was overlaid onto TSA (OV/BEA and OV/EA). Plates were incubated at 37°C for another 22 h, whereas for TAL methods, 14 ml of melted TSA (48°C) was overlaid (TAL/BEA and TAL/EA). Heat-injured cells were plated onto TAL media and then incubated at 37°C for 24 h.

Thermal inactivation study under laboratory conditions. The turkey litter compost with six months of aerobic thermophilic stabilization (plant A) and laying hen litter compost (plant B) were used to evaluate the *E. faecium* as a surrogate for validating thermal processes under laboratory conditions. All the composts were dried under the fume hood until moisture content was reduced to less than 20%, and then screened to the particle size of less than 3 mm using a sieve. Sufficient compost samples were collected for all laboratory experiments and stored in a sealed container at 4°C until use.

The moisture contents of different poultry litter composts were adjusted based on the moisture range of incoming poultry litter compost processed in both plants, i.e. 30-50% for the turkey litter compost received from plant A, and 15% for the laying hen litter compost from plant B. Desiccation-adapted *S.* Senftenberg 775/W or *E. faecium* cultures were prepared as described previously by Wang et al. (22). Following the 24-h desiccation adaptation, the compost with desiccation-adapted cells was further mixed with compost with the same moisture content at a ratio of 1:100 using a sterile blender (KitchenAid Inc., St. Joseph, MI) to the target final concentration of desiccation-adapted cells. In brief, both desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* were inoculated into the turkey litter compost adjusted to 20, 30, or 40% moisture content, and into the laying hen litter compost with 15% moisture content, respectively, at a final concentration of ca. 5 log CFU g^{-1}. Twenty grams of compost in duplicate were distributed evenly inside a sterile aluminum pan (I.D. 10 cm). When the oven temperature reached the set temperature (5°C higher than the target temperature), the aluminum pans with inoculated samples were placed on the oven shelf at two different locations.

According to treatment temperatures in each plant, temperatures used were 75 and 150°C for turkey litter compost and 75°C for laying hen litter compost. Thermal inactivation experiments were performed as described above. For heat treatments at 75°C, compost samples were withdrawn from the oven at 0 min and every 30 min during the come-up time and holding time (up to 180 min) for determining microbial populations. For heat treatment at 150°C, duplicate samples were withdrawn every 15 min up to 60 min.

Specifications of industrial dryers. Two poultry litter compost processors were involved in the plant validation studies: one turkey litter compost processor (plant A) in the midwestern United States, and one laying hen litter compost processor (plant B) in the southwestern United States. Specifically, plant A processes the turkey litter compost with six months of aerobic thermophilic stabilization, whereas plant B process the composted chicken manure mixed with the bone meal (laying hen litter compost). Although the dryers in both plants can bring the treatment temperature to > 65°C, and operated at a 13-22 rpm rotation rate, the dryer in plant A was operated continuously, and the dryer in plant B was batch-operated. The detailed specifications of industrial dryers are listed in Table 1.

Sampler design. Based on our previous composting-related studies and heat tolerance tests, a Tyvek bag was used to hold the poultry litter compost which allowed the moisture transfer but no sample leakage. To protect the Tyvek bag from breaking up by stones and other sharp objects mixed in compost during drying inside the dryer, sampler systems were designed and tested in plant A (Figure 1). Initially, three sampler prototypes (tea

infuser, mesh cylinder, and suet wire basket) were tested in plant A. All prototypes were retrieved immediately from the knockout unit (for trapping the stones) after the dryer. Both the tea infuser and mesh cylinder were torn apart, whereas the suet wire basket (C&S Products Co. Inc., Fort Dodge, Iowa; 14.22 x 12.19 x 4.57 cm) remained intact. By adding stainless steel mesh (2 mm hole; 13.97 x 15.24 x 2.54 cm) as the liner inside the basket, the Tyvek bag remained intact after passing through the dryer at least twice.

Sample preparation for plant validation. One week before each plant trial, poultry litter compost samples were obtained from each processor. The rifampin-resistant *E. faecium* was used as the surrogate microorganism for the plant validation study. The washed overnight culture was added to the turkey litter compost (plant A) or laying hen litter compost (plants B), at a rate of 1:100 vol wt^{-1} to a final concentration of ca. 7 log or ca. 9 log CFU g^{-1}, respectively, and both adjusted to the desired moisture content (within the moisture range of compost from each plant). For plant A, about 50 g of inoculated turkey litter compost was packed into each Tyvek pouch (12.7 × 12.7 cm) with all sides reinforced with heat-tolerant tape. Two Tyvek pouches, one inoculated and one uninoculated, were placed into one customized sampler as described above. For plant trials conducted at plant B, instead of using a sampler, sterilized polyester kitchen cloth swatches (2.54 × 2.54 cm, ca. 80 pieces for each trial, with ca. 20% moisture content) were mixed with ca. 2.46 kg of inoculated laying hen litter compost (ca. 15% moisture content, each trial) to serve as indicators for the mixing and progression through the cooker (residence time) in plant B (Figure 1). Afterward, all the prepared samplers for plant A and the inoculated laying hen litter compost for plant B were shipped overnight at

room temperature to plants A and B, respectively, allowing bacterial cells to be desiccation-adapted in the compost during shipping. Tyvek bags containing poultry litter compost with or without being inoculated with *E. faecium*, in duplicate, served as shipment controls for each trial.

Sample collection and analysis. For trials in plant A, samplers were retrieved at the exit end of the dryer after being dropped into the entry of the dryer. The residence time for each sampler was recorded. Compost samples before and after heat treatment were also collected from the processing line to serve as process controls. For each trial performed in plant B, process control samples were taken from the dryer before the addition of inoculated samples. About 2.46 kg of laying hen litter compost (ca. 15% moisture content) with desiccation-adapted *E. faecium* were mixed with the bulk of compost (ca. 680 kg per run) in the dryer before heat treatment. After each test run, the distribution of the kitchen cloth swatches from the catch bin was observed to determine if the inoculated sample was sufficiently mixed. Meanwhile, 12 samples from different representative locations of the catch bin were collected for sample analysis. Two separate test runs were conducted sequentially for each trial. All collected samples including the shipment controls were shipped overnight with cold packs back to Clemson University, SC, and analyzed immediately.

Bacterial enumeration. The surviving *S.* Senftenberg 775/W cells were enumerated using a modified two-step overlay method (OV/XLT-4-R) to allow heat-injured cells to resuscitate (Chen et al. 2013). The surviving *E. faecium* cells were enumerated using the

recovery media (EA-R). The samples that were presumptively negative for *S.* Senftenberg 775/W by the direct plating method (detection limit: 1.3 log CFU g^{-1}) were screened for *Salmonella* by following the microbiological detection method described by the U.S. FDA Bacteriological Analytical Manual (22). The samples that were presumptively negative for *E. faecium* by the direct plating method were pre-enriched in brain heart infusion broth (BHI) supplemented with 100 µg rifampin ml^{-1} at 37°C for 24 h, followed by plating onto EA-R. Besides, both the turkey litter compost and laying hen litter compost samples were analyzed for indigenous microorganisms, including presumptive enterococci, mesophiles, and thermophiles, and screened for *Salmonella* based on the methods described in the U.S. FDA Bacteriological Analytical Manual (23). For plant A, the indigenous microorganisms were enumerated from both compost sample without inoculation of *E. faecium* in the sampler and the compost sample collected from the processing line. For plant B, the inoculated compost was added on-site prior to the thermal process, and, therefore, to exclude the interference from the inoculated *E. faecium*, the indigenous microorganisms were enumerated from the sample collected from the processing line only (prior to the inoculation of *E. faecium*).

Chemical and physical characteristics analysis. Moisture content was measured with a moisture analyzer (Model IR-35, Denver Instrument, Denver, CO). Water activity (a_w) was measured with a dew-point a_w meter (Aqualab Series 3TE, Decagon Devices, Pullman, WA). The pH and electrical conductivity values of poultry litter compost were measured by a multi-parameter benchtop meter (Orion VERSA Star meter, Thermo Fisher Scientific Inc., Fort Collins, CO, USA) according to the test methods described by

the U.S. Composting Council (24). Duplicate samples were analyzed by the Clemson University Agricultural Service Lab for chemical characteristics, including nutrients and heavy metals (total concentrations, including water-soluble and water-insoluble concentrations).

Statistical analysis. Plate counts were converted to log CFU g^{-1} in dry weight. SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) was used for data analysis. Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was carried out to determine whether significant differences ($P < 0.05$) existed among different treatments. In this study, P-value < 0.05 was used to indicate a strong evidence against the null hypothesis or a statistically significant result. The Spearman's rank-order correlation was calculated using RStudio 1.1.463 (RStudio, Inc., Boston, MA, USA) to determine the strength of association between changes in chemical-physical properties and microbial reductions in poultry litter compost during plant validation studies.

RESULTS AND DISCUSSION

Comparing heat resistance between desiccation-adapted *E. faecium* and *S.* Senftenberg 775/W under laboratory conditions. In order to accurately enumerate heat-injured *E. faecium* and presumptive indigenous enterococci from turkey litter compost, six culturing methods were compared for recovering heat-injured cells of enterococci. Due to the interference from background microorganisms with the similar morphology showing on plates (data not shown), overlay methods (OV/EA, OV/BEA, TAL/EA, and TAL/BEA) were not selected for enumeration of presumptive indigenous

enterococci from heat-treated turkey litter compost. As shown in Table 2, EA and OV/EA-R were selected to enumerate the heat-injured presumptive indigenous enterococci and *E. faecium*, respectively.

To reduce the uncertainty during processing plant-scale studies, laboratory-based studies were performed first to compare the thermal resistance of desiccation-adapted *E. faecium* and desiccation-adapted *S.* Senftenberg 775/W in both turkey litter and laying hen litter composts. No *Salmonella* was detected from the received compost samples. For the laboratory-based study, the come-up times for heating turkey litter compost with 20, 30, and 40% moisture content, and laying hen litter compost with 15% moisture content at 75ºC were 75, 92, 142, and 60 min, respectively. The population reductions of desiccation-adapted *E. faecium* were significantly ($P < 0.05$) lower than those of desiccation-adapted *S.* Senftenberg 775/W under all the tested conditions (Figure 2). At 75ºC, the log reductions of desiccation-adapted *E. faecium* in the turkey litter compost with 20, 30, and 40% moisture content were 1.4, 2.1, and 2.9 log CFU g^{-1}, respectively, after come-up times and holding for 180 min, whereas desiccation-adapted *S.* Senftenberg 775/W was detected by enrichment only (with a 4.2-log reduction) in the turkey litter compost with 20% moisture content during holding times (Figure 2A). More than a 5-log reduction of desiccation-adapted *S.* Senftenberg 775/W was achieved during come-up times of 90 and 120 min in the samples with 30 and 40% moisture contents, respectively (Figures 2B and C). At 150°C, desiccation-adapted *E. faecium* survived for up to 15 min in the turkey litter compost with 20% moisture content, whereas it was not detectable for other moisture contents (data not shown). However, desiccation-adapted *S.* Senftenberg

775/W could not be detected in the turkey litter compost with all moisture contents exposed to 150°C within 15 min (data not shown). In the laying hen litter compost with ca. 15% moisture content (Figure 3), a 1.5-log reduction of desiccation-adapted *E. faecium* was detected after a come-up time of 60 min at 75°C, as compared with > 5-log reduction of desiccation-adapted *S.* Senftenberg 775/W. During the holding time, cell counts of desiccation-adapted *E. faecium* were still more than 4 log CFU g^{-1} in the laying hen litter compost after exposure to 75°C for 180 min, whereas *Salmonella* cells were not detected by enrichment after 90 min.

Taken together, the thermal inactivation effect on both desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* was found to be enhanced with the increase in moisture content of poultry litter compost in the laboratory validation study. This finding was in line with our previously published laboratory-scale studies on the physical heat-treatment of *Salmonella* spp. and its surrogate microorganism in broiler chicken litter compost (4, 22). Based on the laboratory validation studies, reductions of 1.2- to 2.7- log or more of *E. faecium* can predict > 5-log reductions of *S.* Senftenberg 775/W in poultry litter compost, depending on heating temperature, moisture content, and types of poultry litter compost. In short, findings from laboratory studies indicated that *E. faecium* can be used as a surrogate for *Salmonella* to provide a sufficient safety margin when validating the thermal processing in industrial settings.

Sampler design and residence time determination. To ensure the inoculated compost samples could be processed through the industrial dryer in plant A and subsequently

collected post-drying, a sampler was designed to hold the turkey litter compost. To secure the sampler, the suet-wire basket was held tightly with heat-tolerant cable ties (Figure 1). The sampler is easy to assemble on site and thus can be used as a suitable validation tool in industrial settings. For plant A, the residence times for the winter trial (44% moisture content), spring trial (36% moisture content), and summer trial (36 and 44% moisture contents) were 28-198, 23-72, 26-60, and 25-59 min, with the median resident time as 51, 45, 43, and 35 min, respectively, as measured by running the sampler though the dryer.

To our knowledge, only pilot-scale models have been reported so far for animal wastes process validation (26, 27). And for all previous pilot-scale studies on physical heat treatment of animal wastes, only pig slurry with a maximum capacity of 220 liters/h was studied using indigenous microorganisms (28). As compared to those pilot-scale studies, both heat transfer and compost flow are difficult to control in an industrial processing line. As such, the custom-designed samplers were used as validation tools by holding compost samples intact during thermal processing in the industrial dryer for the subsequent plant validation studies.

Plant-scale validation of physical heat-treatment of poultry litter compost using indicator microorganisms. The thermal inactivation rate of presumptive indigenous enterococci was found to have a correlation with that of desiccation-adapted *S.* Senftenberg 775/W in broiler litter with different moisture contents when subjected to heat treatment under laboratory conditions (29). Our intent in this study was therefore to use enterococci, mesophiles, and thermophiles as potential indicator microorganisms to

determine the thermal inactivation rates of indigenous microorganisms in industrial settings. Reductions of indicator microorganisms in plant A are presented in Table 3. An average of > 3-log reduction of presumptive indigenous enterococci from four trials were found in both uninoculated samples in Tyvek bags and the turkey litter compost collected from the processing line. Average population reductions of indigenous mesophiles and thermophiles in processing control samples were 2.2 and 1.1, 2.5 and 1.5, 1.7 and 0.4, and 2.2 and 1.1 log CFU g^{-1} for the winter trial (44% moisture content), the spring trial (36% moisture content), the summer trial 1 (36% moisture content), and the summer trial 2 (44% moisture content), respectively (Figure 4).

For plant B, the initial population level of presumptive indigenous enterococci in the laying hen litter compost was lower than that in the turkey litter compost of plant A. After physical heat treatment of laying hen litter compost in plant B, the population of presumptive indigenous enterococci was reduced > 1.8 log CFU g^{-1} in the processing control samples (Table 4), whereas mesophiles and thermophiles were reduced by 2.6 and 2.5, 2.9 and 3.2 log CFU g^{-1} for trial 1 and 2, respectively (Figure 4). Variations in the initial populations of indigenous microorganisms in incoming poultry litter compost were observed among trials in both plants.

Due to the variable populations of indigenous microflora and heterogeneous compost ingredient in poultry litter compost, multiple microbial indicators, such as enterococci, mesophiles, or thermophiles, could be potentially used to represent a wide spectrum of pathogens existing in compost that have different levels of heat resistance. In agreement

with this suggestion, Cunault et al. (26) used a set of indigenous microorganisms, including naturally occurring *E. coli*, enterococci, spore-forming sulfite-reducing *Clostridia* (SRC), and indigenous mesophiles (MCB), as indicators to assess the effectiveness of the thermal treatment of pig slurry. They reported that a continuous heat treatment at 55 or 60°C for 10 min can reduce 4- or 5- log of indigenous *E. coli,* whereas a longer heat treatment at these temperatures was needed to kill enterococci, and indigenous SRC was more heat-resistant as compared to enterococci in pig slurry treated at 96°C for 10 min. Although there are limited studies on plant-scale validation studies, many lab-based studies have also reported the use of different indigenous microflora, including fecal *Streptococcus*, *Enterococcus* spp., and *E. coli,* as indicator microorganisms to determine the survival behaviors of bacterial pathogens during animal wastes composting or wastewater treatment (30, 31, 32). In the present study, thermal inactivation of indigenous microorganisms depended on the time-temperature combinations and types of poultry litter compost. In consideration of the low cost to enumerate presumptive indigenous enterococci, the processors could do routine monitoring of their thermal process by using indigenous microorganisms as indicators.

Plant-scale validation of physical heat treatment of poultry litter compost using surrogate microorganism. In addition to indicator microorganisms, surrogates could also be an alternative for predicting the survival characteristics of pathogens in plant-scale studies. Due to the fluctuation of presumptive indigenous enterococci populations in animal wastes, the use of spiked *E. faecium* should be considered for validation purpose. As shown in Table 3, only two samples were found positive for *E. faecium* after

enrichment, which were obtained from the samplers exiting from the dryer with the shortest residence times (36 and 28 min, respectively) for the winter trial with 44% moisture content and the summer trial with 36% moisture content, respectively. During the second summer trial with 36% moisture content, there was an 8-min shut-down of the drying process due to a power outage, which might have interfered with the normal movement of samplers inside the dryer and heat treatment.

In plant B, the initial moisture content of the laying hen litter compost was ca. 17% and the initial inoculum level of *E. faecium* was 8.9 log CFU g^{-1}. The *E. faecium* population decreased by ca. 2 log CFU g^{-1} during the 48-h desiccation adaptation at room temperature. For plant B validation, ca. 2.46 kg compost spiked with desiccation-adapted *E. faecium* was mixed with one batch of compost (ca. 680 kg) resulting in an approximate 1:280 ratio (Figure 1). Two separate trials were performed with 4.5 log CFU g^{-1} as the average target population of desiccation-adapted *E. faecium* in laying hen litter compost before heat treatment. After heat processing (ca. 99.4°C for 7 min), the cloth swatches (mixing indicators) were found to be well-distributed, indicating a homogeneous mixing of laying hen litter compost mixing during the drying process. *E. faecium* was detected by direct plating in 3 out of 20 samples, and by enrichment in 14 out of 20 samples. In summary, our data showed that an average > 2.8-3.1-log reduction in surrogate microorganism was achieved after heat treatment in plant B in both trials (Table 4).

Based on the results from the plant validation studies, we confirmed the suitability of using desiccation-adapted *E. faecium* as a surrogate for desiccation-adapted *S.*

Senftenberg 775/W when validating physical heat treatment of poultry litter compost. After a slight decrease in the populations of *E. faecium* in poultry litter compost during shipping, the desiccation-adapted *E. faecium* populations were found to maintain at a stable level before heat treatment, which was in agreement with the requirements that the ideal surrogate should be easy to yield a high-density level with constant population until utilized (33, 20). After physical heat treatment in the plants, the log-reduction of desiccation-adaptation *E. faecium* can predict a > 5-log reduction of desiccation-adapted *S.* Senftenberg 775/W in poultry litter compost sources from two plants based on the results produced from lab validation studies.

Correlation between physicochemical changes and microbial reductions after industrial heat treatments. In our previous lab-based study, *E. faecium* was found more sensitive to high temperature in a relatively wet environment, as indicated by its declining heat resistance with increased moisture content of broiler litter during thermal processing (22). Heat transfer could be more efficient in the poultry litter compost with lower moisture content (10), suggesting that other physicochemical characteristics of poultry litter compost might also affect the heat resistance of bacteria during heat treatment. For example, during chicken manure composting, the inactivation rates of *Salmonella* and *L. monocytogenes* in the compost with 20:1 C:N formulations were higher than in 30:1 and 40:1 formulations (34). As afore-mentioned, in addition to moisture level in compost and processing temperature, the physicochemical characteristics of poultry litter compost could be another important factor affecting the population reductions of indicator and surrogate microorganisms during heat treatments.

In this study, the physicochemical characteristics of turkey litter compost from plant A were significantly different ($P < 0.05$) from those of plant B, except for the carbon value (Table 5). Based on the correlation analysis for the compost samples collected from two plants, there were no noticeable correlations between most of the nutrient content (total nitrogen, carbon, C:N, and organic matter) of poultry litter compost and microbial population reduction due to heat exposure in two industrial dryers. The changes in pH of poultry litter compost were strongly correlated with the reductions in indigenous mesophiles, thermophiles, and enterococci, with correlation coefficients values (ρ) greater than 0.7 (Supplement Table S1). Most importantly, pH changes were significantly ($P = 0.049$) negatively correlated with thermophiles reduction, whereas positively correlated with enterococci reduction ($P = 0.017$), indicating thermophiles were inactivated more at lower pH, and enterococci were inactivated more at higher pH.

Unexpectedly, in plant A, the moisture content of turkey litter compost had no significant effect ($P > 0.05$) on the reductions of the surrogate microorganism. It should be noted that processing plants only process poultry litter compost with a narrow range of moisture content. According to the information provided by plant A, the moisture content of turkey litter compost averaged at 36% with a range of 30-50%. For plant B, the *E. faecium* population in the laying hen litter compost with 15% moisture content was less reduced after heat treatment compared to the higher moisture turkey litter compost with 36 and 44% moisture contents from plant A. This can be explained by the increased heat resistance of microorganisms in the dry matrix (35). As such, the slight difference between the two moisture contents used in plant A trials would not allow the surrogate

microorganism to produce a significant change in heat resistance. Further, although the design of the Tyvek bags allowed the free movement of moisture during heat processing, there was less moisture reduction when compost samples were contained inside the sealed Tyvek bags. Nonetheless, in the processing control samples, the reductions of indicator microorganisms were significantly ($P < 0.05$) affected by the moisture content, as the highest enterococci cell reduction (3.7 log CFU g^{-1}) was achieved in the processing control compost samples with the highest moisture content (51.2%). Therefore, the use of indicator microorganisms may have the added advantage of reflecting changes in the properties of litter compost during heat-processing as compared to surrogate microorganisms.

As discussed above, although a laboratory-based study on the same source compost samples was performed before the plant validation studies, the environment in a plant certainly cannot be controlled as ideally as under laboratory conditions. Therefore, it is important to apply the laboratory findings to real-world use by thoroughly assessing the effectiveness of physical heat-treatment process in an industry setting. When scaling up a physical heat-treatment to a processing plant, the homogeneity and the flow of poultry litter compost in the larger industry dryer should be controlled to ensure a uniform heating of litter compost. The custom-designed sampler was thus able to hold compost samples and surrogate microorganisms intact during the heating processing in the rotary dryer. When designing the plant validation studies, it is important to take into consideration the industrial settings, such as dryer specifications, type of heat treatment, and incoming poultry litter compost handling. For example, different methods were used

to prepare the inoculum of surrogate microorganisms for the two processing plants. Moving from laboratory to plant validation study, physical heat treatments in both processing plants have been scientifically validated as an effective way to reduce potential *Salmonella* contamination in poultry litter compost, which is in line of the FSMA's recommendation of using alternative treatments for reducing or eliminating human pathogens in untreated BSAAO before land application (5). As indicated in Biosolids technology fact sheet published by US Environmental protection agency (US EPA), the dryers used in the participating plants were the most important and common types used for drying biosolid waste in US (36). The validation methods optimized in our research can be applied to other animal wastes-processing plants and provide scientific evidence for the complying to FSMA requirements to produce biological soil amendments safety.

Limitations of this study. There are some limitations to our plant validation studies. Due to the dryer structures of the plants, only end-point samples could be collected. Although many specifications of the dryers were known, some real-time processing data, including come-up times and internal temperatures profile and movement of samples, were not available. Therefore, future studies on the validation of thermal processing using poultry litter compost with various physicochemical properties under industrial settings are necessary.

Conclusions. In summary, for the first time, the thermal processes of two poultry litter compost processing plants under different processing conditions were successfully

validated using *E. faecium* and presumptive indigenous enterococci. Even though the processing conditions in these processing plants varied greatly, the validation results indicated that *Salmonella* levels, if present, could be reduced by at least 5-log based on the reductions of surrogate and indicator microorganisms. The designed sampler could withstand the harsh environments created by high temperatures and strong tumbling movement inside the industrial dryers and served as a carrier for the inoculated poultry litter or poultry litter compost samples exposed to the thermal process in the industry setting. Therefore, both indicator and surrogate microorganisms along with the sampler can serve as practical tools for poultry litter or poultry litter compost processors to routinely monitor and validate their thermal processes without introducing pathogens into the industrial environments.

ACKNOWLEDGMENTS

We thank our industrial collaborators for providing the poultry litter compost samples and supporting the plant validation studies and Dr. Mengzhe Li at the Ocean University of China for assisting us on sample analysis. This research was financially supported by the Center for Produce Safety, CA, and the U.S. Department of Agriculture's (USDA) Agricultural Marketing Service through grant 15SCBGP-CA-0046. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA.

REFERENCES

1. **Ritz CW,** Merka WC. 2009. Maximizing poultry manure use through nutrient management planning. https://athenaeum.libs.uga.edu/handle/10724/12463 Accessed on Dec. 2019.

2. **United States Department of Agriculture Agricultural Marketing Service National Organic Program.** Processed animal manures in organic crop production. 2011. https://www.ams.usda.gov/sites/default/files/media/5006.pdf Accessed on Dec. 2019

3. **Assessment H.** 2015. Commodity specific food safety guidelines for the production and harvest of lettuce and leafy greens. http://www.lgma.ca.gov/wp-content/uploads/2014/09/California-LGMA-metrics-08-26-13-Final.pdf Accessed on Nov. 2019

4. **Chen Z,** Diao J, Dharmasena M, Ionita C, Jiang X, Rieck J. 2013. Thermal inactivation of desiccation-adapted *Salmonella* spp. in aged chicken litter. Appl Environ Microbiol **79:**7013-7020. doi:10.1128/AEM.01969-13 [doi].

5. **Clements D**, Acuña-Maldonado L, Fisk C, Stoeckel D, Wall G, Woods K, Bihn E. 2019 FSMA Produce Safety Rule: Documentation requirements for commercial soil amendment suppliers. https://ucanr.edu/sites/Small_Farms_/files/315707.pdf Accessed on Feb. 2020

6. **Harris LJ,** Berry ED, Blessington T, Erickson M, Jay-Russell M, Jiang X, Killinger K, Michel Jr FC, Millner P, Schneider K. 2013. A framework for developing research protocols for evaluation of microbial hazards and controls during production that pertain to the application of untreated soil amendments of animal origin on land used to grow produce that may be consumed raw. J Food Prot **76:**1062-1084.

7. **Côté C**, Massé DI, Quessy S. 2006. Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries. Bioresour Technol **97**:686-91.

8. **Qi G,** Pan Z, Sugawa Y, Andriamanohiarisoamanana FJ, Yamashiro T, Iwasaki M, Kawamoto K, Ihara I, Umetsu K. Comparative fertilizer properties of digestates from mesophilic and thermophilic anaerobic digestion of dairy manure: focusing on plant growth promoting bacteria (PGPB) and environmental risk. 2018. J. Mater. Cycles Waste Manag. **20**:1448-57.

9. **Graham JP,** Price LB, Evans SL, Graczyk TK, Silbergeld EK. 2009. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Sci Total Environ **407:**2701-2710.

10. **Chen Z,** Wang H, Ionita C, Luo F, Jiang X. 2015. Effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* spp. Appl Environ Microbiol **81:**6883-6889. doi:10.1128/AEM.01876-15.

11. Goyal S, Dhull SK, Kapoor KK. 2005. Chemical and biological changes during composting of different organic wastes and assessment of compost maturity. Bioresour Technol **96**:1584-91.

12. Chen Z, Jiang X. 2017. Selection of indigenous indicator micro-organisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter. J Appl Microbiol **122:**1558-1569.

13. Ceylan, E., and D. A. Bautista. 2015. Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. J. Food Prot. 78: 934-939.

14. Venkitasamy C, Zhu C, Brandl MT, Niederholzer FJ, Zhang R, McHugh TH, Pan Z. 2018. Feasibility of using sequential infrared and hot air for almond drying and inactivation of *Enterococcus faecium* NRRL B-2354. LWT **95**:123-8.

15. Borowski AG, Ingham SC, Ingham BH. 2009. Validation of ground-and-formed beef jerky processes using commercial lactic acid bacteria starter cultures as pathogen surrogates. J. Food Prot **72**:1234-47.

16. Williams P, Leong WM, Ingham BH, Ingham SC. 2010. Lethality of small-scale commercial dehydrator and smokehouse/oven drying processes against *Escherichia coli* O157: H7-, *Salmonella* spp.-, *Listeria monocytogenes*-, and *Staphylococcus aureus*-inoculated turkey jerky and the ability of a lactic acid bacterium to serve as a

pathogen surrogate. Poster presented at the annual meeting of the Institute of Food Technologists 2010 Jul.

17. **Jeong S,** Marks BP, Ryser ET. 2011. Quantifying the performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. J Food Prot **74:**603-609.

18. **Bianchini A,** Stratton J, Weier S, Hartter T, Plattner B, Rokey G, Hertzel G, Gompa L, Martinez B, Eskridge KM. 2014. Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. J Food Prot **77:**75-82.

19. **Kopit LM,** Kim EB, Siezen RJ, Harris LJ, Marco ML. 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. Appl Environ Microbiol **80:**1899-1909. doi:10.1128/AEM.03859-13.

20. **Anderson DG**, Lucore LA. 2012.Validating the reduction of *Salmonella* and other pathogens in heat processed low-moisture foods. Alliance for Innovation & Operational Excellence, Alexandria, VA.

21. **Smith J,** Benedict R, Palumbo S. 1982. Protection against heat-injury in *Staphylococcus* aureus by solutes. J Food Prot **45:**54-58.

22. Wang H, Chen Z, Li M, Greene AK, Jiang X, Wang J. 2018. Testing a nonpathogenic surrogate microorganism for validating desiccation-adapted *Salmonella* inactivation in physically heat-treated broiler litter. J Food Prot **81:**1418-1424.

23. Andrews W, Jacobson A, Hammack T. 2011. Bacteriological analytical manual, chapter 5: *Salmonella*. Silver Spring, MD.

24. U.S. Composting Council. 2002. The test method for the examination of composting and compost (TMECC). https://www.compostingcouncil.org/page/tmecc Accessed on June. 2020.

25. Almond Board of California. 2014. Guidelines for using *Enterococcus faecium* NRRL B-2354 as a surrogate microorganism in almond process validation. https://www.almonds.com/sites/default/files/guidelines_for_using_enterococcus_faecium_nrrl_b-2354_as_a_surrogate_microorganism_in_almond_process_validation.pdf Accessed on June. 2020

26. Cunault C, Pourcher A, Burton C. 2011. Using temperature and time criteria to control the effectiveness of continuous thermal sanitation of piggery effluent in terms of set microbial indicators. J Appl Microbiol **111:**1492-1504.

27. Cunault C, Burton C, Pourcher A. 2013. The impact of fouling on the process performance of the thermal treatment of pig slurry using tubular heat exchangers. J Environ Manage **117:**253-262.

28. Turner C, Williams SM, Burton CH, Cumby TR, Wilkinson PJ, Farrent JW. 1999. Pilot scale thermal treatment of pig slurry for the inactivation of animal virus pathogens. J Environ Sci Health B **34:**989-1007.

29. Chen Z, Jiang X. 2017. Microbiological safety of animal wastes processed by physical heat treatment: an alternative to eliminate human pathogens in biological soil amendments as recommended by the Food Safety Modernization Act. J Food Prot **80:**392-405.

30. Erickson MC, Liao J, Boyhan G, Smith C, Ma L, Jiang X, Doyle MP. 2010. Fate of manure-borne pathogen surrogates in static composting piles of chicken litter and peanut hulls. Bioresour Technol **101:**1014-1020.

31. Klein M, Brown L, Ashbolt NJ, Stuetz RM, Roser DJ. 2011. Inactivation of indicators and pathogens in cattle feedlot manures and compost as determined by molecular and culture assays. FEMS Microbiol Ecol **77:**200-210.

32. Wéry N, Lhoutellier C, Ducray F, Delgenès JP, Godon JJ. Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. 2008. Water Res **42**:53-62.

33. Busta F, Suslow T, Parish M, Beuchat L, Farber J, Garrett E, Harris L. 2003. The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. Compr. Rev Food Sci Food Saf **2:**179-185.

34. Erickson MC, Liao J, Ma L, Jiang X, Doyle MP. 2014. Thermal and nonthermal factors affecting survival of *Salmonella* and *Listeria monocytogenes* in animal manure–based compost mixtures. J Food Prot **77**:1512-8.

35. Leuenberger P, Ganscha S, Kahraman A, Cappelletti V, Boersema PJ, von Mering C, Claassen M, Picotti P. 2017. Cell-wide analysis of protein thermal unfolding reveals determinants of thermostability. Sci **355:**10.1126/science.aai7825. doi:eaai7825 [pii].

36. US Environmental protection agency Biosolids technology fact sheet: heat drying. 2018 https://www.epa.gov/sites/production/files/2018-11/documents/heat-drying-factsheet.pdf Accessed on Nov. 2020

Table 3.1 Summary table for each plant trial

Plant[a]	Dryer specification			Target Moisture content of compost (%)	Performed date	Total samples analyzed after heat treatment	Processing controls collected
	Size (m)	Temperature	Process capacity				
Plant A	3.7 Diameter/ 15.2 length	Inlet: 593ºC Outlet: 65-82ºC	4082.3 kg/h	44	Nov. 16, 2016	22	4
					July 19 & Aug 8, 2017	24	4
				36	April 28, 2017	30	4
					June 29, 2017	24	4
Plant B	0.9 diameter/ca. 2.3 length	65-104ºC	604.8-642.6 kg/h	15	Dec. 13, 2017	24	2

[a] PlantA, processes the turkey litter compost with six months of aerobic thermophilic stabilization.

Plant B, processes the composted chicken manure mixed with bone meal (laying hen litter compost).

Table 3.2 Comparing media for recovering heat-injured *E. faecium* NRRL B-2354 in the composted turkey litter under laboratory condition

Recovery media	*E. faecium* NRRL B-2354 population (log CFU g^{-1}) after exposure to 75ºC for (min)		
	0	30	60
EA-R	8.2±0.1A[a]	4.0±0.3C	< 2[b]
BEA-R	7.3±0.8B	3.3±0.2D	< 2
OV/EA-R	-[c]	5.1±0.3A	< 2
OV/BEA-R	-	4.7±0.5B	< 2
TAL/EA-R	-	4.8±0.2B	< 2
TAL/BEA-R	-	4.2±0.8C	< 2

[a] Data are expressed as means±SD of three trials. Means with different letters in the same column are significantly different ($P < 0.05$).

[b] Detection limit as < 2 log CFU g^{-1}.

[c] -, not tested.

Table 3.3 Inactivation of desiccation-adapted *E. faecium* NRRL B-2354 and presumptive indigenous enterococci in the composted turkey litter after processing through industrial dryer in plant A [a]

Season (Date)	Target Moisture content (%)	n [b]	Before dryer			After dryer		
			Moisture content (%)	*E. faecium* (log CFU g^{-1})	Presumptive indigenous enterococci[d] (log CFU g^{-1})	Moisture content (%)	*E. faecium* (log CFU g^{-1})	Presumptive indigenous enterococci (log CFU g^{-1})
Winter (Nov/16)	44	11	43.8±0.6	7.4±0.1	5.5±0.2	32.3±12.1	+/- (1/11) [e]	2.2±0.1
Process control			51.2±2.3	N.A. [c]	6.0±0.1	3.1±0.6	N.A.	2.3±0.1
Spring (Apr/17)	36	10	36.4±1.0	7.5±0.1	5.4±0.0	14.8±2.7	- [f]	1.9±0.3
Process control			28.7±4.0	N.A.	4.8±0.2	2.8±0.1	N.A.	1.8±0.2
Summer 1 (Jun/17)	36	12	35.2±1.1	7.7±0.0	5.7±0.0	20.5±0.6	+/- (1/12)	1.8±0.0
Process control			29.9±6.2	N.A.	5.2±0.4	4.8±2.0	N.A.	1.8±0.0
Summer 2 (Jul, Aug/17)	44	12	41.8±1.4	7.5±0.0	6.0±0.1	21.9±0.4	-	1.7±0.0
Process control			36.1±3.6	N.A.	4.8±0.9	2.3±0.9	N.A.	1.6±0.0

[a] Two runs were conducted for each plant trial, and the data were expressed as means± SD.
[b] Number of samplers collected from the dryer.
[c] NA, not applicable.
[d] Enterococci counts enumerated on EA plates.
[e] +, Detected by enrichment. The detection limit of directly plating was 1.3 log CFU/g by dry weight.
[f] -, not detected by enrichment.

Table 3.4 Inactivation of desiccation-adapted *E. faecium* NRRL B-2354 and presumptive indigenous enterococci in laying hen litter compost after processing through industrial dryer in plant B

Plant run	Before dryer				After dryer			
	Moisture content (%)	n [a]	*E. faecium* [b] (log CFU g^{-1})	Presumptive indigenous enterococci (log CFU g^{-1}) [d]	Moisture content (%)	n [f]	*E. faecium* (log CFU g^{-1}) [g]	Presumptive indigenous enterococci (log CFU g^{-1}) [h]
Trial 1	6.4±0.0	2	4.5±0.0	N.C. [e]	5.2±0.1	10	< 1.4±0.3	N.C.
Process control	6.3±0.0	2	N.A.[c]	3.6±0.1	2.2±0.0	2	N.A.	< 1.3
Trial 2	6.2±0.0	2	4.5±0.1	N.C.	4.8±0.0	10	< 1.7±0.3	N.C.
Process control	6.3±0.0	2	N.A.	3.1±0.1	2.2±0.0	2	N.A.	< 1.3

[a] The number of samples shipped/collected before each plant trial run.
[b] Expected initial surrogate population prior to dryer, calculated based on mixing-ratio before dryer in plant B.
[c] N.A., not applicable.
[d] Enterococci counts enumerated on EA plates.
[e] N.C. no data collected.
[f] Number of samplers collected from the dryer.
[g] From two trials, 3 out of 20 samples were positive for *E. faecium* NRRL B-2354 by direct plating. Whereas other samples were detected only by enrichment.
[h] Presumptive indigenous enterococci were not detected by directly plating after heat treatment, and the detection limit of direct plating was 1.3 log CFU g^{-1} by dry weight.

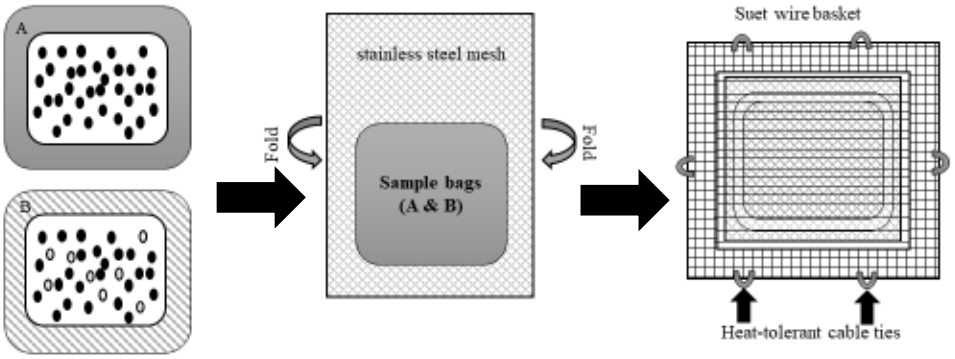
Table 3.5 Chemical-physical characteristics of poultry litter compost of two plants

Sample (Season, Mon/Year)	Before heat treatment					
	Total Nitrogen (%)	Carbon (%)	C:N ratio (%)	Organic matter (%)	EC[b] (mmhos cm^{-1})	pH
Plant A (Winter Nov/16)	3.2±0.1[a]	30.4±0.4	9.4±0.2	58.0±0.3	29.7±1.0	8.5±0.0
Plant A (Spring Apr/17)	4.0±0.0	34.0±0.0	8.4±0.0	63.5±0.4	21.4±0.8	7.6±0.0
Plant A (Summer 1 Jun/17)	2.7±0.1	25.8±0.5	9.4±0.5	48.6±5.3	12.4±1.7	8.8±0.3
Plant A (Summer 2 July, Aug/17)	2.9±0.2	26.8±1.8	9.2±0.0	54.5±4.6	12.5±0.5	8.7±0.0
Average for Plant A	**3.2±0.6A**	**29.2±3.7A**	**9.1±0.5A**	**55.4±6.7A**	**19.0±8.3A**	**8.4±0.5A**
Plant B (Dec/17)	**6.0±0.9B**	**28.9±0.7A**	**5.2±0.1B**	**50.4±1.2B**	**11.9±0.4B**	**6.1±0.0B**
Sample (Season, Mon/Year)	After heat treatment					
	Total Nitrogen (%)	Carbon (%)	C:N ratio (%)	Organic matter (%)	EC (mmhos cm^{-1})	pH
Plant A (Winter Nov/16)	4.7±0.1	37.4±0.7	7.6±0.0	61.9±2.4	35.3±0.2	7.0±0.0
Plant A (Spring Apr/17)	3.9±0.0	34.8±0.2	8.9±0.1	64.6±0.6	20.7±1.2	6.8±0.0
Plant A (Summer 1 Jun/17)	2.8±0.0	25.3±1.4	9.1±0.5	47.8±0.5	13.6±0.9	7.4±0.2
Plant A (Summer 2 July, Aug/17)	2.8±0.2	23.6±1.8	8.4±0.0	44.4±1.7	11.6±1.0	8.2±0.5
Average for Plant A	**3.6±1.0A**	**30.3±6.8A**	**8.5±0.7A**	**54.7±10.0A**	**20.3±10.7A**	**7.3±0.6A**
Plant B (Dec/17)	**5.7±0.1B**	**28.5±5.0A**	**5.000.1A**	**47.8±1.4B**	**11.8±0.4B**	**6.1±0.0B**

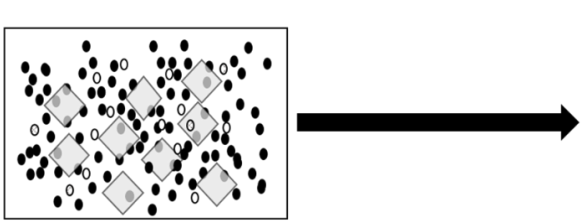
[a] Data are expressed as means±SD of two samples. Means with different letters in the same column are significantly different ($P < 0.05$). The values of nutrients and metals are all calculated based on dry weight.

[b] EC, electrical conductivity.

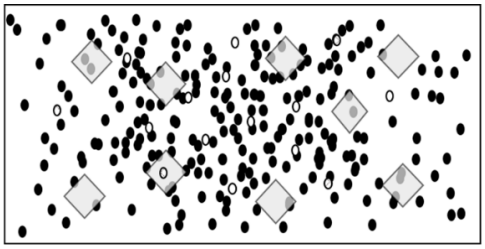
Figure 3.1 Custom-design multiple component sampler (top), and the inoculation procedure for plant B (bottom).



Tyvek bags containing compost without (A) and with (B) inoculated *E. faecium* NRRL B-2354 (the white dots), and sealed with heat-tolerant duct tape.



Laying hen litter inoculated with *E. faecium* NRRL B-2354 (the white dots), followed by mixing with sterilized kitchen cloth swatches (the square shape) as a mixing indicator.



The inoculated laying hen litter mixed with the bulk of laying hen litter in the dryer prior to heat treatment in plan B.

Figure 3.2 Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in the composted turkey litter with 20% (A), 30% (B), and 40% (C) moisture content at 75°C. Inactivation curves during come-up times (to the left of the vertical dotted line) and during holding times (to the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.3 log CFU g^{-1}). Data were expressed from the average of two trials.

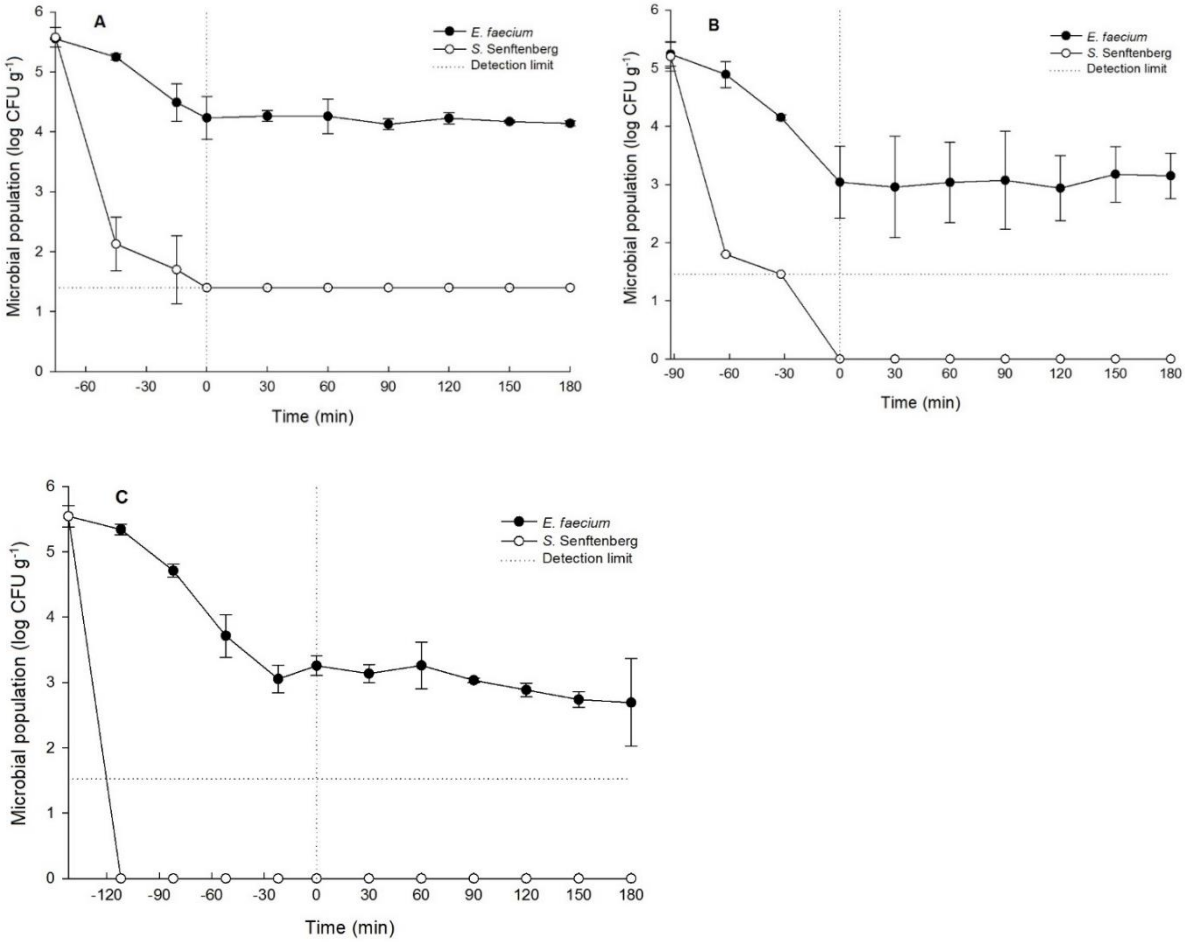


Figure 3.3 Survival of desiccation-adapted *S.* Senftenberg 775/W and *E. faecium* NRRL B-2354 in laying hen litter compost with 15% moisture content at 75°C. Inactivation curves during come-up times (to the left of the vertical dotted line) and during holding times (to the right of the vertical dotted line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.3 log CFU g^{-1}). Data were expressed from the average of two trials.

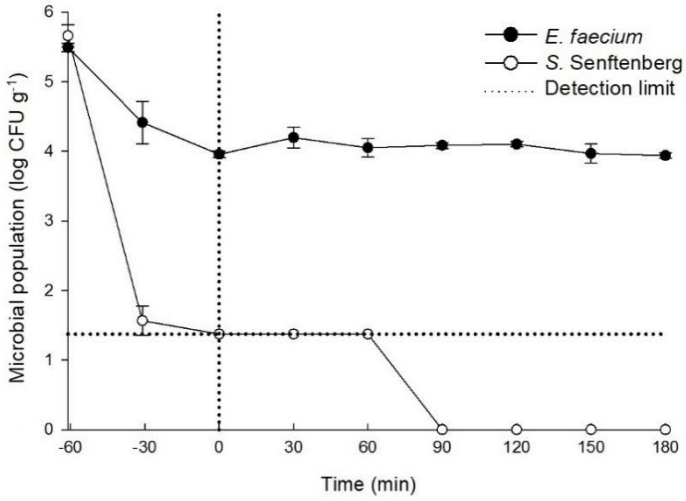
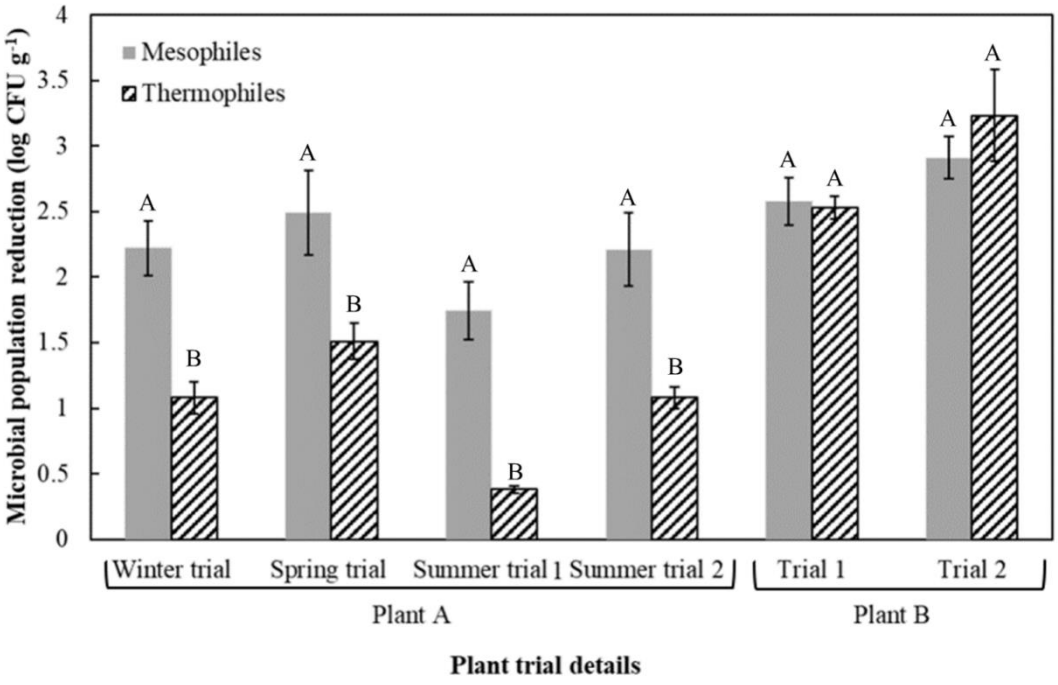


Figure 3.4 The average log reduction of mesophiles and thermophiles in the processing control samples during heat treatment in plants A and B. Means with different letters in the same trial are significantly different ($P < 0.05$) in the reductions between mesophiles and thermophiles.



CHAPTER FOUR

COMPOSITIONAL AND FUNCTIONAL CHANGES IN MICROBIAL COMMUNITIES OF COMPOST DUE TO THE PRESENCE OF *LISTERIA MONOCYTOGENES*

ABSTRACT

In order to understand the complexed interactions between native compost microorganisms and *Listeria monocytogenes*, compost samples collected across the US were subjected to the inoculation of *L. monocytogenes* (ca. 10^7 CFU/g), and then systematically analyzed by 16S rRNA gene, shotgun-metagenomic, and metatranscriptomic sequencing approaches along with culturing methods. The reductions of *L. monocytogenes* in dairy and poultry compost with 40 or 80% moisture content at room temperature after 72 h of incubation ranged from 0.1 to 1.1 log CFU/g, but the regrowth of *L. monocytogenes* occurred in some compost samples after 72 h of incubation, ranging from 0.1 to 1.5 log CFU/g. The major bacterial phyla identified in all farms are *Firmicutes* (23%), *Proteobacteria* (23%), *Actinobacteria* (19%), *Chloroflexi* (13%), *Bacteroidetes* (12%), *Gemmatimonadetes* (2%), and *Acidobacteria* (2%). The statistical analysis of sequencing data revealed that microbial composition and interactions were affected by the environmental factors such as compost types and location, moisture levels and incubation length, rather than the inoculation of *L. monocytogenes*. Although the similarities percentage (SIMPER) results were not significant for all samples, some specific genera (*Bacillus*, *Sphaerobacter*, *Filomicrobium*, *Paucisalibacillus*, *Brumimicrobium*, *Steroidobacter Flavobacterium*, or *Chryseolinea*) were identified as discriminant microorganisms contributing to the variation in community composition due

to the inoculation of *L. monocytogenes* on multiple farms. Besides, after 72 h of incubation, changes in the metabolic pathways and the increased abundance of the bacteriocins category in the compost samples containing *L. monocytogenes* suggest that the interactions between *L. monocytogenes* and compost microbiome may include competition for compost nutrients and the presence of antimicrobials produced by compost microbiome. Findings from this study clearly indicated that microbial diversity and functional profiles were significantly ($P < 0.05$) affected by the compost source, composting stage, and collection farm. Furthermore, the presence of specific discriminant microbial species may suggest certain compost samples as the potential sources for isolating CE microorganisms against *L. monocytogenes*.

INTRODUCTION

Listeria monocytogenes, a leading foodborne pathogen, is ubiquitous in nature, including soils and animal wastes, which makes source control difficult to achieve. Importantly *L. monocytogenes* poses a major public health risk due to its ability to thrive in both farming and food processing environments (Guerra et al., 2001, Gholipour et al., 2020). A key to develop mitigation strategies against this pathogen is to understand its ecology. Animal wastes used in compost production is considered as a nutrient-rich and complex ecosystem that contains diverse groups of microorganisms. During composting, the microbial activities in this ecosystem can generate moderate levels of heat to achieve pathogen reduction (Gurtler et al., 2017). Compost microbial community may also carry out suppressive activities that affect a variety of plant and human pathogens (Sidhu et al.,

2001, Mulero-Aparicio et al., 2020). Therefore, the complex interactions among enteric human pathogen and the indigenous microbiome may determine the fate of enteric pathogen in pre- or post-harvest environments.

One of the most effective and environmental friendly biological control methods is competitive exclusion (CE) (Mead et al., 2000, Hibbing et al., 2010). The host-pathogen interactions have been extensively reported in the animal intestinal ecosystem (De Jong et al., 2012, Ji et al., 2017) and many studies have concluded that microbial diversity is a key factor in reducing pathogen outbreaks (Vivant et al., 2013, Tan et al., 2019). Several beneficial microorganisms with antagonistic activities against soilborne pathogens were identified from compost (Ren et al., 2012, Al-Ghafri et al., 2020). For example, Al-Ghafri et al. (2020) demonstrated that *Pythium aphanidermatum* and *Fusarium solani,* originally isolated from horse litter-based compost, possessed antagonistic activity against *Pseudomonas aeruginosa* during co-culturing studies. However, the ecology and functionality of naturally occurring antagonistic microorganisms found in biological soil amendments is less studied.

Next-generation sequencing approaches have become a powerful tool for characterizing the structure, functional capabilities, and activities of complex microbial communities. In general, 16S rRNA gene (or rDNA) sequencing has enabled us to understand the taxonomic composition of a microbiome, whereas the shotgun-metagenomic sequencing can provide more comprehensive information because it sequences genes from all microorganisms presented in a sample (Cao et al., 2017). The microbial composition and the functional capacity of microbial communities during the

composting process has been documented in several studies using DNA-based sequencing approaches (Wei et al., 2018, Wang et al., 2018, Liu et al., 2020). However, a major limitation of genomic DNA sequencing analysis is the inability to differentiate live (dormant cells as well as growing or non-growing metabolically active cells) and dead cells. To avoid the extraction of DNA from dead cells, investigators used a pre-treatment viability assay with propidium monoazide (PMA) before DNA extraction (Li et al., 2017). Therefore, DNA-based sequencing combined with PMA treatment can theoretically analyze DNA from living cells only.

Metatranscriptome profiling, the deep sequencing of the mRNAs derived from complex microbial communities, allows for characterization of the genes under expression within a complex and diverse population. Analyzing the metagenome adds an additional taxonomic signature to amplicon-based community profiling. Metatranscriptomic sequencing has been previously used by Antunes et al. (2016) to characterize the dynamics of microbial interactions and the role of microbial enzymes in degradation of biomass in compost samples. Their results demonstrated that biomass degradation during composting is fully performed by bacterial enzymes, possibly derived from *Clostridiales* and *Actinomycetales*. To date, there is a paucity of data and in-depth investigations that use high throughput-sequencing to examine the microbiota of commercial animal waste-based compost products (Neher et al., 2013, Pandey et al., 2018). In addition, there is a lack of information on how the compost microbiota responds to the intrusion of pathogens of public health concern, such as *L. monocytogenes*.

To fill these research gaps, the microbial community of commercial compost products was analyzed with and without *L. monocytogenes* using next-generation sequencing approaches. The objective of this chapter was to reveal the composition and functional capabilities of compost microbiome in a variety of composts in the presence and absence of *L. monocytogenes*. Ultimately, findings from this study may assist with discovering and isolating compost-adapted competitive exclusion (CE) microorganisms that could be applied to other environments, such as produce growing and processing environments, to control *L. monocytogenes*.

MATERIALS AND METHODS

Compost sample collection. A total of 12 biological soil amendments (6 dairy- and 6 poultry waste-based composts) were collected from 6 different facilities. Those facilities were located in Arizona, California, Michigan, South Carolina (n=2), and Wisconsin, United States. Each facility provided composting samples at two stages within the composting process: thermophilic composting stage (active compost, > 55°C /131°F, within 1 month of composting) and finished stage (finished compost, 3 to 6 months of composting). Major compost ingredients included green waste, organic animal manure, dairy sawdust, or cow paunch. Following the sampling protocol recommended by the California Leafy Greens Marketing Agreement (LGMA, 2010), samples were collected in Ziploc bags, shipped under the ambient condition to our lab, and stored at refrigeration conditions (4°C) once received. Importantly, as a precaution against potential freeze-damage to cell membranes, compost samples were deliberately not frozen. To reduce the

DNA degradation and microbial population change, the sample preparation and microbiological analysis for most samples were performed within 10 days after the samples were received.

Physicochemical and microbiological analyses of biological soil amendments. Compost samples were analyzed for total aerobic bacteria, actinomyces, yeast/mold, *Enterobacteriaceae*, thermophilic and heterotrophic bacteria by plating serial dilutions onto 3M™ Petrifilm™ aerobic count plates (3M, USA), Actinomycete Isolation Agar (AIA; Becton Dickinson and Company; NJ, USA), Rose Bengal Agar (RBA; Hardy Diagnostic; CA), Violet Red Bile Glucose Agar (VRBG; Hardy Diagnostic), tryptic soy agar (TSA; BD) and Reasoner's 2A agar (R2A; BD), respectively, followed by incubation at 35°C for 24 h, 25°C for 48 h, 25°C for 5 days, 35°C for 24 h, 55°C for 24 h and 25°C for 5 – 7 days, respectively. Samples were also examined for the presence of background *L. monocytogenes* by following Food and Drug Administration's Bacteriological Analytical Manual (Hitchins et al., FDA-BAM) procedure and enumerated onto Oxford agar (Hardy Diagnostic). DNA was extracted from the black colonies grown on Oxford agar (Difco, BD, Sparks, MD) and confirmed by polymerase chain reaction assay that targeted the *hly*A gene (Soni et al., 2014).

Moisture contents of compost samples were measured with a moisture analyzer (model IR-35, Denver Instrument, Denver, CO), whereas pH values were measured based on the methods described by U.S. Composting Council (2002). Additionally, compost samples in duplicate were analyzed by Clemson Agricultural Service Laboratory for

chemical characterisation, including total nitrogen, carbon, organic matter, and soluble salts.

Compost inoculation and *L. monocytogenes* enumeration. The experimental designs for compost inoculation and sequencing analysis are shown in Figure 4.1. Each composite compost sample was divided into three portions to serve as technical replicates for the subsequent experiments. The compost samples were thoroughly mixed and adjusted to 40 or 80% moisture contents with autoclaved tap water and half of the samples were artificially inoculated with *L. monocytogenes* strain FSL R9-5506 (a pathogenic strain isolated from the packaged salad, kindly provided by Dr. Martin Wiedmann at Cornell University). To prepare for the inoculum, the *L. monocytogenes* culture was streaked twice onto TSA, and then grown overnight in tryptic soy broth (TSB) at 35°C, followed by washing and resuspending in 0.85% saline to ca. 10^9 CFU/ml. Afterwards, the culture was inoculated into the compost samples with the target moisture contents (40 or 80%) at a final inoculation level of ca. 7 log CFU/g. At 0 and 72 h post inoculation, *L. monocytogenes* population in each compost sample was enumerated by plating 10-fold serial dilutions, in duplicate, onto Oxford media plates, followed by incubation at 35°C for 24 h.

DNA and RNA extraction. Although there was regrowth of *L. monocytogenes* occurred in compost samples collected from dairy farm #1, the discriminant microbial genera (potential CE) were more abundant in this compost sample. Besides, the dairy farm #1 was able to provide the compost samples from two collections with the same ingredients and composting length (active compost), which could be considered as biological replicates. Therefore, the fresh compost samples from dairy farm #1 were collected for the

shotgun-metagenomic and metatranscriptomic sequencing studies. As shown in Figure 4.1, DNA was extracted from all samples and used for 16S rRNA gene sequencing, whereas fresh active compost samples were requested from dairy farm #1 prior to shotgun-metagenomic and metatranscriptomic sequencing. The total DNA and RNA were extracted using the ZymoBIOMICS DNA and DNA/RNA miniprep extraction kits (Zymo Research, Irvine, CA, USA), respectively, according to manufacturer's instructions. *DNase I* treatment step was included during RNA extraction for DNA removal. For PMA treatment (Fittipaldi et al., 2012, Li et al., 2017), the compost slurry (1:4 w/v) was transferred to a transparent 2-ml microcentrifuge tube and mixed with propidium monoazide (PMA dye, Biotium, Inc. CA, USA) at a final concentration of 50 μM in a dark room and incubated for 5 min on a rotating mixer at room temperature. Next, the microcentrifuge tube was subsequently placed on ice horizontally and exposed to 650 W halogen light source at 20 cm distance for 20 min. Afterwards, PMA was removed by centrifugation at 12,000 × *g* for 5 min and DNA was extracted immediately from the compost samples.

RNA was further purified using the RNA Clean & Concentrator kit (Zymo Research, Irvine, CA). The purity and concentration of both DNA and RNA were evaluated on a NanoDrop -2000 spectrophotometer (NanoDrop Technologies, DE, USA) at 260, 280, and 230 nm. Further quantification of DNA and RNA was performed using Qubit fluorometer (Thermo Fisher, MA, USA). RNA integrity number (RIN) was determined using an RNA 6000 Nano kit in the 2100 Bioanalyzer (Agilent Technologies, USA). Upon completion of extraction, samples were submitted to the respective sequencing facilities for 16S rRNA gene, shotgun-metagenomic, or metatranscriptomic sequencing analysis.

16S rRNA gene sequencing and sequencing data processing. In total, 288 gDNA samples were submitted to ZymoBIOMICS Services (Zymo Research, Irvine, CA) for library preparation and bacterial 16S rRNA gene sequencing. Bacterial 16S rRNA gene targeted sequencing was prepared using the Quick-16S NGS library prep kit. The bacterial 16S primers were used to amplify the V3-V4 region of the 16S rRNA gene. These primers were custom-designed by Zymo Research to provide the best coverage of the 16S gene while maintaining high sensitivity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator (Zymo Research, Irvine, CA), then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit. The final library was sequenced on Illumina MiSeq with a Version 3 reagent kit (600 cycles). The sequencing was performed with >10% PhiX spike-in.

After high-throughput 16S rRNA gene sequencing, paired-end sequence reads were joined together. Unique amplicon sequences were inferred from raw reads and chimeric sequences were removed using the DADA2 pipeline (Callahan et al., 2016). Following further size filtration, the chimera-free sequences for each sample were analyzed through Qiime default pipeline (Kuczynski et al., 2011). After further fragment size filtration, the average number of chimera-free sequences for each sample was 55,420 ± 18,610 for further analysis (Table 4.1). Taxonomy annotation was performed using Uclust from Qiime V.1.9.1 with the Zymo Research database (Caporaso et al., 2010), a 16S database that is internally designed and curated, as reference. Before further analysis, the raw counts of each taxonomy were adjusted for sequencing depth by even sampling and for 16S copy number variation in order to represent true relative abundances (Kembel et al., 2012). The

16S copy number adjustment was performed using copy number estimates from the Ribosomal RNA Database (rrnDB) v5.4.

Shotgun-metagenomic and metatranscriptomic sequencing. In total, 24 DNA and 8 RNA samples were submitted to Novogene Inc. (Sacramento, CA) for shotgun-metagenomic and metatranscriptomic sequencing, respectively. Sequencing libraries were generated using NEBNext DNA Library Prep Kit (New England Biolabs, Ipswich, MA) following the manufacturer's recommendations, and indices were added to each sample. For the metatranscriptomic sequencing, after the initial QC procedure, mRNA from eukaryotic organisms was enriched using oligo (dT) beads. Simultaneously, rRNA was removed using the Ribo-Zero kit (Illumina, San Diego, CA) and the mRNA was concentrated. Following the QC steps, the qualified libraries from DNA and RNA were fed into Illumina sequencers (PE 150) after pooling according to its effective concentration and expected data volume, respectively.

To get clean reads for both shotgun-metagenomic and metatranscriptomic sequencing, reads containing adapter and low-quality base (Q-score ≤ 5) were removed from the raw reads. Afterwards, reads were further filtered and trimmed with AfterQC (a tool for automatic filtering, trimming, error removing, and quality control for fastq data) (Chen et al., 2017). All the shotgun-metagenomic and metatranscriptomic sequencing data had a > 99% “good reads” after filtering. Afterwards, all reads were uploaded into the MG-RAST analysis sever (https://www.mg-rast.org/), which provides an open source server made up of a high-throughput pipeline built for high-performance computing of metagenomes (Keegan et al., 2016). Paired reads were combined and subjected to quality

filtering, and host sequences were depleted. The default parameters of the MG-RAST were used for the taxonomic and functional assignation of the sequences. All the Illumina reads that were shorter than 75 bases or had a median quality score below 20 were removed. The functional annotation was based on the SEED hierarchical system or KEGG database.

Statistical analyses. Plate count data were converted to log CFU/g in dry weight. The Shapiro-Wilk test of normality was run to test for normalcy statistically prior to sequencing data analysis. The non-parametric analysis methods were used for the non-normal distribution data set. For the 16S rRNA gene sequencing data analysis, within-community diversity (alpha diversity) was calculated by Chao richness and Shannon index of species using Qiime V.1.9.1, followed by Kruskal-Wallis and Wilcoxon singed-rank tests. Beta (β)-diversity, variation of microbial communities between environmental samples, was measured with ecological phylogenetic Unifrac distances (Lozupone et al., 2011). Prior to the analysis, the relative abundance data set at the genus level was subjected to chord transformation to account for many zero values (Legendre et al., 2018). Linear discriminant analysis (LDA) effect size (LEfSe) was applied to search for biomarkers between different compost types (Segata et al., 2011). Principle-coordinate analysis (PCoA) was performed to determine whether samples associated with the same groups (compost type or composting stage, experimental moisture, incubation time, presence or absence of *L. monocytogenes*) clustered close to one another in multivariate space. Permutational multivariate analysis of variance (PERMANOVA) was used to test the statistical significance of group separation in PCoA with Benjamini–Hochberg False Discovery Rate adjusted *P* value (BH-FDR). Canonical correspondence analysis (CCA)

was used to explicitly test whether the different experimental factors explained a significant fraction of the variation within the distance matrix. Lastly, the similarity percentage (SIMPER) was used to find key taxa contributing to the variation in community composition due to the presence of *L. monocytogenes.* These analyses were run in R with packages including vegan and phyloseq (Dixon et al., 2003, McMurdie et al., 2015).

For shotgun-metagenomic and metatranscriptomic sequencing data analysis, statistical comparisons of the proportions of functions among treatment groups of samples were conducted using STAMP software (Statistical Analysis of Shotgun-metagenomic Profiles; Parks et al., 2014). Briefly, an ANOVA test was used to compare among multiple groups, followed by Tukey–Kramer post hoc tests. White's non-parametric t-test with Benjamini–Hochberg FDR correction for multiple tests were used for comparing two groups of data, and Welch's inverted method was used to calculate 95% confidence intervals. ROTS was used to provide a rank of the functional gene expression based on their differential change due to the presence of *L. monocytogenes* after 72 h of incubation. Then, the expression of functional genes with significant fold changes (FDR P-value < 0.05) in each sample was visualized by a heatmap (Suomi et al., 2017).

RESULTS

Survival of *L. monocytogenes* and cultivable microorganisms in compost samples. The population of total cultivable aerobic bacteria, heterotrophs, thermophiles, *Enterobacteriaceae*, yeast/mold, and actinomycetes in the collected compost samples ranged from approximately 6.8 to 9.7, 5.6 to 8.9, 3.3 to 8.6, < 2.1 to 6.1, < 2.1 to 6.3, and

5.7 to 8.7 log CFU/g, respectively (Table 4.2). For all categories, relatively higher levels of total aerobic bacteria, heterotrophs (except for poultry farm #3), and thermophiles were observed for the active compost as compared to the finished compost collected from the same farm. The population level of yeast/mold was found to be significantly ($P < 0.05$) lower than the population of cultivable bacterial species. After selective enrichment, there were some black colonies observed on Oxford agar, but those black colonies were then confirmed as non-*L. monocytogenes* with PCR targeting the *hlyA* gene.

The reductions of *L. monocytogenes* in compost at room temperature after 72 h of incubation ranged from 0.1 to 1.1 log CFU/g (Table 4.3), whereas there was regrowth of *L. monocytogenes* of 0.1 to 1.5 log CFU/g occurred in the compost samples mainly with 80% moisture content from poultry farm #2 and dairy farm #1 regardless of composting stage.

Taxonomic distributions of compost microbial communities in different compost types. To further explain the differential clustering of microbial communities from compost samples, the taxonomic composition of four types (active dairy, finished dairy, active poultry, and finished poultry) of compost samples were examined without *L. monocytogenes* inoculation. Overall, the major bacterial phyla observed in all farms were *Firmicutes* (23%), *Proteobacteria* (23%), *Actinobacteria* (19%), *Chloroflexi* (13%), *Bacteroidetes* (12%), *Gemmatimonadetes* (2%), and *Acidobacteria* (2%), accounting for 94% of sequences in all compost samples. At phyla level, the relative abundance of each phylum representing >1% of the total taxonomy, and *Chloroflexi* represented a significantly greater proportion of reads in finished dairy compost, followed by

Proteobacteria and *Firmicutes* in finished poultry compost, *Actinobacteria* in active poultry compost, *Firmicutes* and *Proteobacteria* in active dairy compost (Figure 4.2A). At the genus level, LEfSe was used to detect microbial genera that differed significantly between each compost type and different genera for which the LDA score was > 2 were considered significant biomarkers for that group. As indicated by LEfSe's output (Figure 4.2B), the top 3 indicator genera (biomarkers) enriched in finished poultry, active poultry, finished dairy, and active dairy composts were *Chryseolinea*, *Hyphomicrobium* and *Planctomyces*; *Brachybacterium*, *Salinicoccus* and *Brevibacterium*; *Aerolinea*, *Altererythrobacter* and *Thioalkalispira*; and *Ureibacillus*, *Cellvibrio* and *Idiomari*, respectively. While for the experimental factors, clear distinction between the compost samples with 40 and 80% moisture contents was visible, and this difference was more pronounced after 72 h incubation (Figure 4.3). There was no direct evidence that taxonomic compositions in each farm were affected by the inoculation of *L. monocytogenes*.

Microbial diversity in compost samples (α-diversity). Chao richness and Shannon indices were used to show microbial species richness and diversity in compost samples. The diversity and richness differed significantly (BH-FDR adjusted *P*-values < 0.05) among the compost types as indicated by the non-parametric Kruskal-Wallis test (Figure 4.4). Regardless of composting stages, dairy farm #1 and poultry farm #2 had the lowest microbial diversity based on Chao richness index, for both *L. monocytogenes* inoculated and uninoculated samples. Among the experimental variables, α-diversity indices were significantly affected by moisture content and incubation time, but not by *Listeria* inoculation according to Wilcoxon signed-rank test (Table 4.4). The farms that

have the lowest diversities (Figure 4.4) also had the greater proliferation (> 0.5 log CFU/g) of spiked *L monocytogenes* at higher moisture content (Table 4.3).

Variation in microbial compositions of composts (β-diversity). The distances from the β-diversity of microbial communities among farms were visualized in the exploratory principle-coordinate analysis (PCoA) plot (Figure 4.5). Clustering was analyzed using a weighted UniFrac distance matrix, which incorporates phylogenetic relatedness among 16S rRNA gene sequences when calculating distance. The variances of 61.1% and 19.9% in the β-diversity were explained by the PC1 and PC2 for microbial composition in dairy compost, respectively, whereas the variances of 57.3% and 14.5% in the β-diversity were explained by the PC1 and PC2 for microbial composition in poultry compost, respectively. The PCoA plots showed that the microbial composition of compost samples collected from each farm formed a distinct cluster, while for each farm, microbial composition of active compost samples appeared to be more distinct from those of finished compost. Overall, for all farms, the microbial compositions of compost samples were not separated by the experimental factors (moisture contents, incubation length, and the presence of *L. monocytogenes*; Figures 4.5 B, C, D, F, G, H). This observation was consistent among dairy (Figures 4.5A) and poultry farms (Figures 4.5E).

In line with the PCoA ordination, PERMANOVA showed that the composition of the microbiota varied significantly by composting farm with BH-FDR adjusted *P*-values < 0.05 (Figures 4.5A and E). However, all the experimental factors had no significant effects on the compositions of microbiota with BH-FDR adjusted *P*-values > 0.05. In addition, the effects of experimental variables on microbial composition in each farm were studied by

canonical correspondence analysis (CCA) with the three experimental conditions (moisture, incubation length, and presence of *L. monocytogenes*) as constraining variables. The variation in microbial compositions of compost samples from dairy farms #1 and #3, and poultry farm #2 were significantly associated with both incubation time and experimental moisture (BH-FDR adjusted $P < 0.05$), whereas the microbial composition of compost samples from poultry farm #3 was only affected by incubation time (BH-FDR adjusted $P < 0.05$) (Table 4.5). It is noteworthy that the total dissimilarity due to *L. monocytogenes* was a small percentage of the overall variations, which could be inferred from χ^2 residual that was calculated from the CCA model.

SIMPER analysis. SIMPER was used to identify the microbial members that separate *L. monocytogenes*-inoculated and uninoculated communities in different composts. As shown in Figure 4.6, the top 20 discriminant microbial members (Bray-Curtis dissimilarity, contribution to dissimilarity percentage ranged from 1 - 10%, not significant) were identified. However, the discriminatory genera identified by SIMPER did not significantly ($P > 0.05$) contribute to the differences in compost microbial communities in the presence or absence of *L. monocytogenes*. As indicated from the results of PCoA and CCA analysis, the total dissimilarity due to the introduction of *L. monocytogenes* has a very small percentage of the overall variations. It needs to point out that the responses (increase or decrease) from these dissimilarity genera and *L. monocytogenes* varied in different compost samples, as indicated by the different color codes in bubble plots. When applying SIMPER to samples collected from each farm, *Bacillus*, *Sphaerobacter*, *Filomicrobium*, *Paucisalibacillus*, *Brumimicrobium*, *Steroidobacter Flavobacterium*, or

Chryseolinea were separately identified as microbial members that contributed to a small percentage (Bray-Curtis dissimilarity, 1 - 10%) of the variations that separate *L. monocytogenes*-inoculated and uninoculated communities on multiple farms. Moreover, those discriminant microorganisms were highly abundant in compost samples collected from dairy farm #1 (Figure 4.7). Therefore, fresh compost samples were requested from dairy farm #1 for the subsequent shotgun-metagenomics and metatranscriptomic sequencing.

Microbial community composition and functional capacities of compost without *L. monocytogenes* as revealed by shotgun-metagenomic sequencing analysis. As revealed by shotgun-metagenomic sequencing, active dairy compost was dominated by bacteria (98.14%), followed by archaea (0.97%), eukaryote (0.53%), virus (0.01%), and other unassigned sequences (< 0.01%). For the taxonomic results of bacteria, shotgun-metagenomic and 16S rRNA gene sequencing results agree with each other. The five most abundant phyla in active dairy compost collected from dairy farm #1 are *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes,* and these five phyla account for at least 91% of all classified reads in active dairy compost samples (data not shown).

The shotgun-metagenomic sequencing also provided insights into the metabolic potential of organisms inhabiting in the collected compost sample. The predicted proteins were annotated using the SEED subsystems (Level 1). Clustering-based subsystems and carbohydrate metabolism had the largest quantity of annotated reads assigned in the active dairy compost samples, representing 15 and 13%, respectively (Figure 4.8). To gain a

comprehensive understanding of the functional capacities of active dairy compost from farm #1, two separated collections were performed from two composting rows with the same ingredients and composting length. Based on SEED subsystem level 1, 8 of the 28 functional profiles were found to be significantly different (BH-FDR adjusted $P < 0.05$) between two collections (Figure 4.9). The function profiles associated with DNA metabolism and motility and chemotaxis were found to be higher in relative proportions in collection A as compared to collection B, whereas fatty acids, lipids, and isoprenoids were higher in relative proportions in the collection B.

Comparison of the microbial functional capacities with and without *L. monocytogenes* by shotgun-metagenomic sequencing analysis. After 72 h of incubation, there was ca. 0.5 log CFU/g regrowth of *L. monocytogenes* in the compost samples freshly collected from dairy farm #1 with 80% moisture content. The microbial functional profiles of composts, with and without inoculation of *L. monocytogenes* as classified at SEED functional gene entries, were further analyzed separately for the two collections at 72 h incubation. For collection B, genes that are assigned to different functional roles including controlling pyoverdine biosynthesis, anaerobic sulfite reductase subunit, L-ascorbate utilization, and divergent RNA modification related cluster (HD family hydrolase) were significantly (BH-FDR adjusted $P < 0.05$) enriched in compost samples inoculated with *L. monocytogenes*, whereas meiosis-specific DNA cleavage protein was found to be more abundant in proportions in the compost samples without *L. monocytogenes*. These observations were consistent among the technical replicates in collection B (Figure 4.10). Overall, for collection B, the core functional capacities at subsystem level 2 of compost

microorganisms changed due to *L. monocytogenes* inoculation belong to different subsystem level 1 categories, including iron acquisition and metabolism, DNA metabolism, respiration, and carbohydrate metabolism. However, there was no such trend observed in collection A. The inoculation of *L. monocytogenes* did not induce the change in the aforementioned functional capacities of compost microbiome from collection A. These results were not surprising because the functional profiles of compost samples from these two collections were statistically different.

Microbial gene expression patterns in compost with and without *L. monocytogenes* as revealed by metatranscriptomic sequencing analysis. A multiple-group comparison was used to identify the pathways whose gene expression was not equal across all treatment groups (compost sample inoculated with or without *L. monocytogenes* at 0 or 72 h incubation periods). Overall, 75 of 196 pathways of active functional categories were found at the SEED subsystem level 2 from multiple-group comparison. This suggests the means of proportions of these pathways from all treatment groups were not equal. Next, we used a post-hoc test to identify which pairs of groups differ from each other and found that the mean proportions in some pathways significantly increased due to inoculation of *L. monocytogenes* after 72 h incubation ($P < 0.05$). Only selected categories with BH-FDR adjusted $P < 0.001$ are presented in Table 4.6. In contrast, at 0 h post inoculation, the mean proportions of the above pathways in compost samples with or without *L. monocytogenes* did not differ significantly ($P \geq 0.05$).

After 72 h incubation, in total, 43 genes significantly associated with the inoculation of *L. monocytogenes* were expressed (Figure 4.11A) [BH-FDR corrected *P*-

value < 0.05, ROTS test with rare categories (n < 5 genes) were removed from the analysis]. SEED-annotated genes were later clustered into 23 SEED subsystems level 2 and 17 SEED subsystem level 1, based on SEED hierarchical clustering. Gene expression associated with virulence, disease and defense (SEED level 1) such as bacteriocins, ribosomally synthesized antibacterial peptides, and resistance to antibiotics and toxic compounds were expressed relatively higher in the compost sample inoculated with *L. monocytogenes* as compared to the sample without *L. monocytogenes*. NADH dehydrogenase subunit 4 (EC:1.6.99.3) and negative regulator of genetic competence were the most strongly expressed genes in compost samples with *L. monocytogenes*. The actively expressed genes related to ABC transporter ATP-binding protein (bacteriocins, ribosomally synthesized antibacterial peptides), arsenic efflux pump protein (resistance to antibiotics and toxic compounds), and negative regulator of genetic competence (DNA uptake, competence) were mapped to *Bacillus* spp., *Staphylococcus haemolyticus*, and *Geobacillus* spp., respectively. And the NADH dehydrogenase subunit 4 function was mapped into multiple species of bacteria (Figure 4.11B).

Based on the pairwise testing of the total functional genes annotated with the KEGG database, 117 of the 4414 functional genes expressed were significantly different between compost samples, with and without *L. monocytogenes* inoculation, given a sufficiently small *P*-value (BH-FDR adjusted $P < 0.05$). Among the 117 genes, 56 were associated with metabolism at level 1, including amino acid metabolism; biosynthesis of other secondary metabolites; carbohydrate metabolism, energy metabolism, lipid metabolism,

metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, etc. (Figure 4.11C).

DISCUSSION

Considering the diversity of the compost microbiomes, one can assume that many indigenous species likely interact with *L. monocytogenes* to some extents. However, very little is known about composting ecology and there is no concrete conclusion on how the indigenous microbial community responds to the introduction of *L. monocytogenes* in dairy or poultry composts. In this study, microbial community structures and functions of 12 animal waste-based compost samples were analyzed using high-throughput sequencing approaches. Additionally, the compositional and functional changes of microbial communities in compost due to the environmental factors (composting length/types, compost farms, and moisture contents) and the presence of *L. monocytogenes* were also analyzed at a whole community level.

Impact of cultivable compost microorganisms on the survival of *L. monocytogenes*. In this study, a high-level of *L. monocytogenes* (ca. 7 log CFU/g) was inoculated into the compost samples in order to understand the changes in *L. monocytogenes* populations in different compost samples. *L. monocytogenes* was reduced in most compost samples, but the regrowth of this pathogen occurred in both dairy farm #1 and poultry farm #2 samples. For the finished compost sample collected from poultry farm #2, the pathogen regrowth might be due to the much lower populations in total bacterial counts, heterotrophs, thermophiles, and actinomyces. As for compost samples collected

from dairy farm #1, the low counts of heterotrophs and actinomyces were also observed. In addition to the culturable microorganisms, the interactions from the microbiome with invading pathogens could come from those uncultured compost microorganisms, which account for > 90% of total microorganisms in the ecosystem (Ramamurthy et al., 2014). Studies have been conducted to elucidate that the role of indigenous compost microorganisms in suppression of human pathogens including *L. monocytogenes*, *Salmonella*, and *E. coli* O157: H7 (Zaleski et al., 2005, Kim et al., 2009 & 2010). Kim et al. (2010) reported that the regrowth of *E. coli* O157: H7, *Salmonella* spp., and *L. monocytogenes* was suppressed in dairy manure compost (40% moisture content) by an indigenous microbiota of 6.5 log CFU/g, as compared with the regrowth of 2.1- to 3.9- log in the autoclaved compost. In food processing facilities, the microbiota of *Listeria*-colonized and *Listeria*-free drains and apple washing conveyor belt were characterized as different, indicating the occurrence of *Listeria* was closely associated with the total background microbiota in these built environments at a whole community level (Fox et al., 2014; Tan et al., 2019).

The variance in microbial communities and function profiles of animal waste-based composts. Overall, the most abundant phyla and orders of compost microbes agreed with those found in previous studies (Ryckeboer et al., 2003, Storey et al., 2015, Antunes et al., 2016). Both 16S rRNA gene and shotgun-metagenomic sequencing analysis were revealed that the dominant abundances of *Firmicutes* and *Actinobacteria* were observed in active dairy compost. Significant differences in microbial communities exist among compost types due to the ingredients, composting stage, and the environments of

composting. In active dairy and poultry composts, the primary indicator genera were assigned as *Salinicoccus* and *Ureibacillu*s, respectively, which belong to *Firmicutes*. As most species in *Firmicutes* are more adapted to the thermophilic conditions (Wang et al., 2018, Li et al., 2019), there was higher relative abundance of *Firmicutes* observed in active compost as compared to those in finished compost in most compost samples collected in this study. However, *Firmicutes* was the most abundant phylum in the finished compost collected from poultry farm #2. This inconsistent observation in poultry farm #2 was likely due to the fact that the temperature of the finished compost pile in this farm was still very higher (53.3°C) compared to the temperature of the finished compost samples collected from other farms, highlighting the variation in composting practice in the real world.

In the finished compost, the primary indicator genera belong to *Bacteroidetes*, *Planctomycetes*, *Chloroflexi,* and *Proteobacteria* phyla, with *Chloroflexi* representing a significantly greater proportion of sequence reads in finished dairy compost compared with other types. It was consistent in other studies that the abundance of *Chloroflexi* increased during the maturation phase (Zhong et al., 2018, Li et al., 2019). The increase in abundance of *Chloroflexi* and *Proteobacteria* in the finished dairy compost may also be attributed to their ability to decompose lignocellulose, cellulose, lignin, and other complex organic compounds in dairy manure (Antunes et al., 2016, Ren et al., 2016). Furthermore, the indicator genera *Chryseolinea*, *Hyphomicrobium*, and *Planctomyces*, were found in finished poultry compost. These genera were identified previously in composted chicken manure (Ye et al., 2016, Song et al., 2020), which are known to be involved in N cycling or positively correlated with the presence of NO_3^-. Additionally, it is not surprising that

the microbial composition was unique to each compost farm (Figure 4.3), given that the compost ingredients and composting practice used on each farm were different (Cai et al., 2018). Knowing the fact that the compost-related factors are the major drivers that affected the microbial compositions in compost, presumably, the variance in microbial communities and function profiles of animal waste-based compost was due to the heterogeneous nature of compost samples.

Interactions between *L. monocytogenes* and compost microbiota composition. The sequence reads of *L. monocytogenes* generated by the 16S rRNA gene sequencing were well correlated (R^2 = 0.69) with the number of inoculated *L. monocytogenes* (ca. 7 log CFU/g), which provided the confidence when analyzing the effect of *L. monocytogenes* on the compost microbial composition by sequencing methods. The influence on the transformation of the compost microbial composition from the spiked *L. monocytogenes* was not as strong as that from moisture content or incubation length. Based on these results, we hypothesized that the stability of highly complex microbial communities in compost ecosystem resisted the microbial compositional change for the intrusion of *L. monocytogenes*. In support of this statement, Falardeau et al. (2018) found that native bacterial communities in soil were not driven by the inoculation of *L. monocytogenes* but by differences in pH and moisture contents of natural soil samples. In other studies, effects from inoculated *Salmonella* (ca. 7 log CFU/g) and *E. coli* O157: H7 (ca. 8 log CFU/g) on the composition of the prokaryotic communities were not visible in the untreated-sandy, clay, or regular soil samples (Xing et al., 2020, Schierstaedt et al., 2020). As expected, Schierstaedt et al. (2020) and Xing et al. (2020) reported that the effect of *Salmonella* on

the microbial community was observed only in the autoclaved soils, and phylogenetic diversity decreased by 43.6% due to the inoculated *E. coli* O157:H7 inoculation in irradiated soils. Schierstaedt et al. (2020) also found that the changes in abundance of *Proteobacteria* in autoclaved soil due to *Salmonella* inoculation were decreased over incubation time. Manipulating an environmental microcosm (e.g. irrigation, dilution to extinct, or autoclaving) causes a severe reduction on the microbial diversity and the interactions with invading pathogens. Moreover, the native community was markedly reduced and simplified due to these manipulations. In our study, compost microbial composition without any manipulation was not perturbed by the spiked *L. monocytogenes*, implying that the microbial ecology with complex microbial compositions such as animal waste-based compost, cannot be easily changed by one species of invading pathogen. And the indigenous compost microorganisms also can work against the changes in microbial structures caused by inoculated pathogenic bacteria (Partanen et al., 2010, Zhong et al., 2020). Due to influences from the heterogeneous mixtures of compost samples on the native microbiome, the next reasonable assumption is that the response from compost native microbiome to the introduction of *L. monocytogenes* could be specific to their habitable environments.

Although the presence of *L. monocytogenes* has very limited impact on compost microbiome, the compost diversity showed significant effect on the fate of this pathogen. Based on data collected in the present study, the lower microbial diversities in dairy farm #1 and poultry #2 possible led to the increase in the population of *L. monocytogenes* after 72 h incubation, especially in the compost with higher moisture content, despite the

different sources and collect locations for the samples. Studies have shown that the high species diversity of a mice's gut, fruit processing facilities, and rhizosphere communities was a powerful biological regulator that decreased infectious pathogens (Hu et al., 2016, Ji et al., 2017, Tan et al., 2019). Similarly, Tan et al. (2019) investigated the link between the occurrence of *L. monocytogenes* and the built environment microbiota in three tree fruit processing facilities and reported that a notable higher *L. monocytogenes* was found in one facility with lower microbial diversity. Taken together, these observations suggest that the microbial diversity is a major biological huddle for environmental ecosystems against pathogenic microorganisms (Hu et al., 2003, Tan et al., 2019). As illustrated above, high microbial diversity in compost can prevent the proliferation of *L. monocytogenes*. The low diversity results in low stability of the community composition and increases the probability of perturbation by many stimuli, including the invasion of pathogens. Nonetheless, for all compost samples used in this study, both high levels of compost microorganisms and an adequate inoculation of *L. monocytogenes* can promote the possibility of microbial interaction, shared nutrient and joint secretions in the compost ecosystem (Bauer et al., 2018). Thus, even in the compost sample with relative lower microbial diversity, the competition between native compost microbiome and invading *L. monocytogenes* can be expected. Surely, a better understanding is needed on how the compost microbial communities changed by different factors, such as composting farms, compost types, and experimental factors.

Response from compost microbiome to the presence of *L. monocytogenes* as affected by the environmental factors. According to the stress-gradient hypothesis,

microbial interactions shifted from cooperation to competition as in the nutrients decreased environments (Velez et al., 2018, Hammarlund et al., 2019). We therefore expected the metabolic activities changed between invading *L. monocytogenes* and indigenous microbial members in the compost ecosystems. It could be inferred from the bubble plot (Fig. 4.6) that the response from the native microbiome to *L. monocytogenes* was influenced by the composts' environmental variables. This scenario was primarily due to the variance in nutrients provided by different composts, resulting in a difference in the physiological status of the native microbial members and *L. monocytogenes*. Similar to our findings, Gulis et al. (2003) reported that microbial isolates with antagonistic activity against fungi exhibited a difference in response to the changes in microcosm containing higher level of inorganic nutrients. In a study conducted by Sharma et al. (2020), a bioinoculant containing *Azotobacter chroococcum*, *Bacillus megaterium*, and *Pseudomonas fluorescens* was created to inhibit the survivability of *L. monocytogenes* in *vitro* in Hoagland's medium with the presence of *Cajanus cajan* or *Festuca arundinacea* as plant rhizosphere models. It was observed that the impact of this bioinoculant on the populations of *L. monocytogenes* was highly dependent on the conditions of the rhizosphere model created in the Hoagland's medium. In our study, due to this farm-specific response from the native compost microbiome, *L. monocytogenes* could not have a significant role in shaping the structure of compost community. However, some discriminant microbial members identified by SIMPER analysis like *Bacillus* contain competitive exclusion species reported previously for controlling *L. monocytogenes* in TSB broth, soft agar plates and fresh-cut melon. These CE species included *Bacillus* YD1,

Bacillus sp. *cryopeg*, and *Bacillus psychrodurans* (Liao et al., 2009, Collazo et al., 2017, Das et al., 2017). Considering of the highly abundant of these discriminant members in dairy farm #1, we therefore have focused on this sample as potential source of CE isolation in Chapter V.

Functional potentials and gene expression associated with bacteriocin synthesized and various metabolic pathways changed due to the presence of *L. monocytogenes*. As revealed by metatranscriptomic sequencing, the most strongly enriched gene category in the compost samples inoculated with *L. monocytogenes* was NADH dehydrogenase subunit 4 (EC:1.6.99.3), which is a critical regulator to maintain homeostasis in most bacteria (Ruhland et al., 2019). Although the expression of NADH dehydrogenase was not mapped specific to *L. monocytogenes*, we hypothesized that this gene expression may respond to the pathogen intrusion as the expression of NADH dehydrogenase is one of the important redox-responsive regulators in *L. monocytogenes* or other Gram-positive bacteria to survive in the stressed environment (Ruhland et al., 2019). And negative regulator of genetic competence was another strongly expressed gene in compost sample spiked with *L. monocytogenes*. Based on the mapping results, the negative regulator of genetic competence was associated with *Geobacillus* spp, which suggests a potential competitive activity from *Geobacillus* spp. against *L. monocytogenes*. Consistent with this observation, Ottesen et al. (2016) reported that *Geobacillus* spp. outcompeted with *L. monocytogenes* in ice cream during 4 – 12 h of enrichment. Moreover, in agreement with the results from 16S rRNA genes sequencing, the increased gene abundance in bacteriocins upon the introduction of *L. monocytogenes* suggests that *Bacillus* spp. or other

species from active compost might have antagonistic activity against the invading pathogen by producing antimicrobial peptides. However, a regrowth of *L. monocytogenes* was observed in the compost sample used for metagenomic sequencing after 72 h of incubation. Competitive exclusion by *Geobacillus* spp. against *L. monocytogenes* might only be strong enough to suppress the growth of *L. monocytogenes* initially. Indeed, a similar observation of *L. monocytogenes* overgrowth in ice cream was also seen by Ottesen et al. (2016) after the initial inhibitory period.

According to shotgun-metagenomic sequencing results, different functional roles responsible for the nutrient uptake and carbohydrate utilization (Kentache et al., 2016) were enriched in compost samples inoculated with *L. monocytogenes*. For instance, the dissimilatory sulfite reductase may be related to the environmental variables (Leavitt et al., 2015). In brief, the observations from shotgun-metagenomic sequencing and the changes in the metabolic pathways in compost samples containing *L. monocytogenes* have confirmed that the microbial interactions were potentially affected by the competition for nutrients within the compost environment.

LIMITATIONS

While comprehensive, our research has some limitations. In light of the sample size used for high-throughput sequencing analysis, the experimental design varied among three approaches. Specifically, the extracted RNA was pooled from multiple extractions in order to reach the required concentration. Therefore, the results from metatranscriptomic sequencing should be interpreted with caution due to the insufficient biological replicates.

In general, shotgun metagenomic sequencing is preferred if the research is focused on both taxonomic and functional information. On the other hand, the targeted sequencing would be a more cost-efficient choice for profiling the microbial community in a large-scale project. Further, metatranscriptomic sequencing is used to learn how a microbial community responds to their changing environmental conditions. However, considering the challenges in extraction of high-quality RNA from complex matrix, the large-scale application of metatranscriptomic sequencing of compost samples is currently limited. For a further study, more biological replicates and deeper sequencing should be incorporated in the experimental design as the ability of NGS techniques to detect the minor changes of key microbial species or functional genes in a complex ecosystem is directly dependent on the depth of sequencing and consistent response.

CONCLUSION

The microbial diversity, structure and functions varied among compost samples and were significantly affected by the composting-related factors. To our knowledge, this is the first study that provided a comprehensive analysis of compost microbiome at both composition and function levels. Besides, the interactions between *L. monocytogenes* and indigenous compost microbiome was studied for the first time by high-throughput sequencing approaches. Our study illustrated that the interactions between *L. monocytogenes* and the native microbial members were generally limited and did not affect the dominant members of the microbial community in the compost ecosystem, but some discriminatory species were identified. Metatranscriptomic sequencing has provided the

active gene expression levels associated with bacteriocin production, and therefore can be used to monitor how these levels change when *L. monocytogenes* presented in the compost. Findings from this study can be useful for the composting industry to understand the composition and functionality of microbial community in their products better, and to isolate those discriminatory microbial members as potential competitive exclusion microorganisms from compost samples, and some of which could be leveraged as natural deterrents in the organic fertilizer or produce industry.

ACKNOWLEDGMENT

We thank our industry collaborators for providing the compost samples used in this study. This research was financially supported by the Center for Produce Safety, University of California at Davis, and Specialty Crop Block Grant Program (SCBGP) of California Department of Food and Agriculture (CDFA–SCBGP) through a grant 1#8-0001-075-SC. We also acknowledge the assistance of Ms. Jaime Randise from the Clemson University Genomics and Bioinformatics Facility for services and facilities provided. This facility is supported by Grant P20GM109094 an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health.

REFERENCES

1. Al-Ghafri HM, Velazhahan R, Shahid MS, Al-Sadi AM. Antagonistic activity of *Pseudomonas aeruginosa* from compost against *Pythium aphanidermatum* and *Fusarium solani*. Biocontrol Science and Technology. 2020 Apr 11:1-7.

2. Antunes LP, Martins LF, Pereira RV, Thomas AM, Barbosa D, Lemos LN, Silva GM, Moura LM, Epamino GW, Digiampietri LA, Lombardi KC. Microbial community structure and dynamics in thermophilic composting viewed through metagenomics and metatranscriptomics. Scientific Reports. 2016 Dec 12;6:38915.
3. Bauer MA, Kainz K, Carmona-Gutierrez D, Madeo F. Microbial wars: competition in ecological niches and within the microbiome. Microbial cell. 2018 May 7;5(5):215.
4. Cai L, Gong X, Sun X, Li S, Yu X. Comparison of chemical and microbiological changes during the aerobic composting and vermicomposting of green waste. PloS one. 2018 Nov 26;13(11):e0207494.
5. California leafy greens marketing agreement 2010. Accessed on Mar. 2020 https://lgma-assets.sfo2.digitaloceanspaces.com/downloads/Appendix_E.pdf
6. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods. 2016 Jul;13(7):581.
7. Cao Y, Fanning S, Proos S, Jordan K, Srikumar S. A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. Frontiers in Microbiology. 2017 Sep 21;8:1829.
8. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010 May;7(5):335.

9. Chen S, Huang T, Zhou Y, Han Y, Xu M, Gu J. AfterQC: automatic filtering, trimming, error removing and quality control for fastq data. BMC Bioinformatics. 2017 Mar;18(3):91-100.
10. Collazo C, Abadias M, Aguiló-Aguayo I, Alegre I, Chenoll E, Viñas I. Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7 against food-borne pathogens in vitro and on fresh-cut melon. LWT-Food Science and Technology. 2017 Nov 1;85:301-8.
11. Das G, Park S, Baek KH. Diversity of endophytic bacteria in a fern species *Dryopteris uniformis* (Makino) Makino and evaluation of their antibacterial potential against five foodborne pathogenic bacteria. Foodborne Pathogens and Disease. 2017 May 1;14(5):260-8.
12. De Jong HK, Parry CM, van der Poll T, Wiersinga WJ. Host–pathogen interaction in invasive salmonellosis. PLOS Pathog. 2012 Oct 4;8(10):e1002933.
13. Dixon P. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science. 2003 Dec;14(6):927-30.
14. Falardeau J, Walji K, Haure M, Fong K, Taylor G, Ma Y, Smukler S, Wang S. Native bacterial communities and *Listeria monocytogenes* survival in soils collected from the Lower Mainland of British Columbia, Canada. Canadian Journal of Microbiology. 2018;64(10):695-705.
15. Fittipaldi M, Nocker A, Codony F. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. Journal of Microbiological Methods. 2012 Nov 1;91(2):276-89.

16. Fox EM, Solomon K, Moore JE, Wall PG, Fanning S. Phylogenetic profiles of in-house microflora in drains at a food production facility: comparison and biocontrol implications of *Listeria*-positive and-negative bacterial populations. Applied and Environmental Microbiology. 2014 Jun 1;80(11):3369-74.
17. Gholipour S, Nikaeen M, Farhadkhani M, Nikmanesh B. Survey of *Listeria monocytogenes* contamination of various environmental samples and associated health risks. Food Control. 2020 Feb 1;108:106843.
18. Guerra MM, McLauchlin J, Bernardo FA. *Listeria* in ready-to-eat and unprocessed foods produced in Portugal. Food Microbiology. 2001 Aug 1;18(4):423-9.
19. Gulis V, Suberkropp K. Interactions between stream fungi and bacteria associated with decomposing leaf litter at different levels of nutrient availability. Aquatic Microbial Ecology. 2003 Jan 7;30(2):149-57.
20. Gurtler JB. Pathogen decontamination of food crop soil: a review. Journal of Food Protection. 2017 Sep;80(9):1461-70.
21. Hammarlund SP, Harcombe WR. Refining the stress gradient hypothesis in a microbial community. Proceedings of the National Academy of Sciences. 2019 Aug 6;116(32):15760-2.
22. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology. 2010 Jan;8(1):15-25.

23. Hitchins AD, Jinneman K, Chen Y. FDA BAM Chapter 10: Detection of *Listeria monocytogenes* in foods and environmental samples, and enumeration of *Listeria monocytogenes* in foods.
24. Hu J, Wei Z, Friman VP, Gu SH, Wang XF, Eisenhauer N, Yang TJ, Ma J, Shen QR, Xu YC, Jousset A. Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. MBio. 2016 Dec 30;7(6).
25. Hu JY, Fan Y, Lin YH, Zhang HB, Ong SL, Dong N, Xu JL, Ng WJ, Zhang LH. Microbial diversity and prevalence of virulent pathogens in biofilms developed in a water reclamation system. Research in Microbiology. 2003 Nov 1;154(9):623-9.
26. Ji ZH, Ren WZ, Gao W, Hao Y, Gao W, Chen J, Quan FS, Hu JP, Yuan B. Analyzing the innate immunity of NIH hairless mice and the impact of gut microbial polymorphisms on *Listeria monocytogenes* infection. Oncotarget. 2017 Dec 5;8(63):106222.
27. Keegan KP, Glass EM, Meyer F. MG-RAST, a metagenomics service for analysis of microbial community structure and function. In Microbial environmental genomics (MEG) 2016 (pp. 207-233). Humana Press, New York, NY.
28. Kembel SW, Wu M, Eisen JA, Green JL. Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance. PLoS Computational Biology. 2012 Oct;8(10).
29. Kentache T, Milohanic E, Cao TN, Mokhtari A, Aké FM, Pham QM, Joyet P, Deutscher J. Transport and catabolism of pentitols by *Listeria monocytogenes*. Journal of Molecular Microbiology and Biotechnology. 2016;26(6):369-80.

30. Kim J, Jiang X. The growth potential of *Escherichia coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. Journal of Applied Microbiology. 2010 Dec;109(6):2095-104.
31. Kim J, Luo F, Jiang X. Factors impacting the regrowth of *Escherichia coli* O157: H7 in dairy manure compost. Journal of Food Protection. 2009 Jul;72(7):1576-84.
32. Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Current Protocols in Bioinformatics. 2011 Dec;36(1):10-7.
33. Leavitt WD, Bradley AS, Santos AA, Pereira IA, Johnston DT. Sulfur isotope effects of dissimilatory sulfite reductase. Frontiers in Microbiology. 2015 Dec 24;6:1392.
34. Legendre P, Borcard D. Box–Cox-chord transformations for community composition data prior to beta diversity analysis. Ecography. 2018 Nov;41(11):1820-4.
35. Li R, Tun HM, Jahan M, Zhang Z, Kumar A, Fernando WD, Farenhorst A, Khafipour E. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. Scientific Reports. 2017 Jul 18;7(1):1-1.
36. Li X, Shi XS, Lu MY, Zhao YZ, Li X, Peng H, Guo RB. Succession of the bacterial community and functional characteristics during continuous thermophilic

composting of dairy manure amended with recycled ceramsite. Bioresource Technology. 2019 Dec 1;294:122044.

37. Liao CH. Control of foodborne pathogens and soft-rot bacteria on bell pepper by three strains of bacterial antagonists. Journal of Food Protection. 2009 Jan;72(1):85-92.
38. Liu H, Huang Y, Duan W, Qiao C, Shen Q, Li R. Microbial community composition turnover and function in the mesophilic phase predetermine chicken manure composting efficiency. Bioresource Technology. 2020 Jun 10:123658.
39. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. The ISME journal. 2011 Feb;5(2):169-72.
40. McMurdie PJ, Holmes S. Shiny-phyloseq: Web application for interactive microbiome analysis with provenance tracking. Bioinformatics. 2015 Jan 15;31(2):282-3.
41. Mead GC. Prospects for 'competitive exclusion'treatment to control *Salmonellas* and other foodborne pathogens in poultry. The Veterinary Journal. 2000 Mar 1;159(2):111-23.
42. Mulero-Aparicio A, Trapero A, López-escudero FJ. A non-pathogenic strain of *Fusarium oxysporum* and grape marc compost control *Verticillium wilt* of olive. Phytopathologia Mediterranea. 2020 Mar 14;59(1):159-67.

43. Neher DA, Weicht TR, Bates ST, Leff JW, Fierer N. Changes in bacterial and fungal communities across compost recipes, preparation methods, and composting times. PloS one. 2013 Nov 21;8(11):e79512.

44. Ottesen A, Ramachandran P, Reed E, White JR, Hasan N, Subramanian P, Ryan G, Jarvis K, Grim C, Daquiqan N, Hanes D. Enrichment dynamics of *Listeria monocytogenes* and the associated microbiome from naturally contaminated ice cream linked to a listeriosis outbreak. BMC microbiology. 2016 Dec;16(1):1-1.

45. Pandey P, Chiu C, Miao M, Wang Y, Settles M, Del Rio NS, Castillo A, Souza A, Pereira R, Jeannotte R. 16S rRNA analysis of diversity of manure microbial community in dairy farm environment. PloS one. 2018 Jan 5;13(1):e0190126.

46. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics. 2014 Nov 1;30(21):3123-4.

47. Partanen P, Hultman J, Paulin L, Auvinen P, Romantschuk M. Bacterial diversity at different stages of the composting process. BMC microbiology. 2010 Dec 1;10(1):94.

48. Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Frontiers in Public Health. 2014 Jul 31;2:103.

49. Ren G, Xu X, Qu J, Zhu L, Wang T. Evaluation of microbial population dynamics in the co-composting of cow manure and rice straw using high throughput sequencing analysis. World Journal of Microbiology and Biotechnology. 2016 Jun 1;32(6):101.

50. Ren X, Zhang N, Cao M, Wu K, Shen Q, Huang Q. Biological control of tobacco black shank and colonization of tobacco roots by a *Paenibacillus polymyxa* strain C5. Biology and Fertility of Soils. 2012 Aug 1;48(6):613-20.
51. Ruhland BR, Reniere ML. Sense and sensor ability: redox-responsive regulators in *Listeria monocytogenes*. Current Opinion in Microbiology. 2019 Feb 1;47:20-5.
52. Ryckeboer J, Mergaert J, Vaes K, Klammer S, De Clercq D, Coosemans J, Insam H, Swings J. A survey of bacteria and fungi occurring during composting and self-heating processes. Annals of Microbiology. 2003 Jan 1;53(4):349-410.
53. Schierstaedt J, Jechalke S, Nesme J, Neuhaus K, Sørensen SJ, Grosch R, Smalla K, Schikora A. *Salmonella* persistence in soil depends on reciprocal interactions with indigenous microorganisms. Environmental Microbiology. 2020 Mar 3.
54. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome biology. 2011 Jun;12(6):1-8.
55. Sharma R, Gal L, Garmyn D, Bisaria VS, Sharma S, Piveteau P. Evidence of biocontrol activity of bioinoculants against a human pathogen, *Listeria monocytogenes*. Frontiers in Microbiology. 2020 Mar 11;11:350.
56. Sidhu J, Gibbs RA, Ho GE, Unkovich I. The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. Water Research. 2001 Mar 1;35(4):913-20.
57. Song Z, Massart S, Yan D, Cheng H, Eck M, Berhal C, Ouyang C, Li Y, Wang Q, Cao A. Composted chicken manure for anaerobic soil disinfestation increased the

strawberry yield and shifted the soil microbial communities. Sustainability. 2020 Jan;12(16):6313.

58. Soni DK, Singh M, Singh DV, Dubey SK. Virulence and genotypic characterization of *Listeria monocytogenes* isolated from vegetable and soil samples. BMC microbiology. 2014 Dec 1;14(1):241.
59. Storey S, Chualain DN, Doyle O, Clipson N, Doyle E. Comparison of bacterial succession in green waste composts amended with inorganic fertilizer and wastewater treatment plant sludge. Bioresource Technology. 2015 Mar 1;179:71-7.
60. Suomi T, Seyednasrollah F, Jaakkola MK, Faux T, Elo LL. ROTS: An R package for reproducibility-optimized statistical testing. PLoS Computational Biology. 2017 May 25;13(5):e1005562.
61. Tan X, Chung T, Chen Y, Macarisin D, LaBorde L, Kovac J. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. Microbiome. 2019 Dec 1;7(1):115.
62. U.S. Composting Council. (2002) The test method for the examination of composting and compost (TMECC). Bethesda, MD. https://www.compostingcouncil.org/page/tmecc Accessed on June. 2020.
63. Velez P, Espinosa-Asuar L, Figueroa M, Gasca-Pineda J, Aguirre-von-Wobeser E, Eguiarte LE, Hernandez-Monroy A, Souza V. Nutrient dependent cross-kingdom interactions: fungi and bacteria from an oligotrophic desert oasis. Frontiers in Microbiology. 2018 Aug 6;9:1755.

64. Vivant AL, Garmyn D, Maron PA, Nowak V, Piveteau P. Microbial diversity and structure are drivers of the biological barrier effect against *Listeria monocytogenes* in soil. PLoS One. 2013a Oct 8;8(10):e76991.

65. Wang K, Yin X, Mao H, Chu C, Tian Y. Changes in structure and function of fungal community in cow manure composting. Bioresource Technology. 2018 May 1;255:123-30.

66. Wei H, Wang L, Hassan M, Xie B. Succession of the functional microbial communities and the metabolic functions in maize straw composting process. Bioresource Technology. 2018 May 1;256:333-41.

67. Xing J, Sun S, Wang H, Brookes PC, Xu J. Response of soil native microbial community to *Escherichia coli* O157: H7 invasion. Environmental Pollution. 2020 Jun 1;261:114225.

68. Ye J, Zhang R, Nielsen S, Joseph SD, Huang D, Thomas T. A combination of biochar–mineral complexes and compost improves soil bacterial processes, soil quality, and plant properties. Frontiers in Microbiology. 2016 Apr 8;7:372.

69. Zaleski KJ, Josephson KL, Gerba CP, Pepper IL. Survival, growth, and regrowth of enteric indicator and pathogenic bacteria in biosolids, compost, soil, and land applied biosolids. J. Residuals Sci. Technol. 2005 Jan 1;2(1):49-63.

70. Zhong XZ, Li XX, Zeng Y, Wang SP, Sun ZY, Tang YQ. Dynamic change of bacterial community during dairy manure composting process revealed by high-throughput sequencing and advanced bioinformatics tools. Bioresource Technology. 2020 Mar 3:123091.

71. Zhong XZ, Ma SC, Wang SP, Wang TT, Sun ZY, Tang YQ, Deng Y, Kida K. A comparative study of composting the solid fraction of dairy manure with or without bulking material: performance and microbial community dynamics. Bioresource technology. 2018 Jan 1;247:443-52.

Table 4.1 16S rRNA gene sequencing read processing table for each compost type

Compost source	Composting stage	Rawseqs (R1+R2)	Trimmed_seqs (R1+R2)	Chimera_free_seqs	Unique_seqs	Seqs (after_size_filtration)
Dairy	Active	152352	145697	64500	566	63806
	Finished	123430	117070	52887	701	51765
Poultry	Active	148585	142117	64378	568	63701
	Finished	104849	100250	43543	658	42410

Table 4.2 Microbiological analysis of poultry and dairy compost samples collected from 6 farms

Farms	Compost type	Plate count (log CFU/g) [a]					
		Total aerobic bacteria	Heterotrophs	Thermophiles	*Enterobacteriaceae*	Yeast/Mold	Actinomycetes
Dairy #1	Active	9.7±0.1a[b]	7.4±0.1d	8.3±0.1ab	4.3±0.0d	3.1±0.1c	7.5±0.1b
	Finished	7.8±0.1d	7.0±0d	7.7±0.1b	< 2.1±0e [c]	< 2.1±0d	6.7±0d
Dairy #2	Active	9.5±0a	8.9±0a	7.2±0.1bc	2.3±0e	3.1±0.1c	8.7±0a
	Finished	8.7±0c	8.6±0.1ab	6.6±0.0d	5.1±0c	< 2.3±0d	7.6±0.1b
Dairy #3	Active	9.2±0ab	8.3±0b	8.6±0.0a	6.1±0a	< 2.2±0d	7.7±0.1b
	Finished	8.5±0c	7.9±0c	7.4±0.1b	6.1±0.1a	< 2.2±0d	7.6±0.1b
Poultry #1	Active	9.0±0b	8.4±0.1b	7.5±0.1b	5.7±0.1b	5.6±0b	6.9±0.1d
	Finished	7.8±0.1d	7.7±0.1c	7.1±0.1c	5.7±0b	6.3±0.1a	7.2±0.1c
Poultry #2	Active	8.3±0.1c	7.1±0d	5.4±0.0e	< 2.1±0e	3.3±0.1c	8.3±0.1ab
	Finished	6.8±0.1e	5.6±0e	3.3±0.0f	2.2±0.1e	< 2.1±0	5.7±0.1e
Poultry #3	Active	8.1±0.1c	7.2±0d	7.4±0.0b	4.9±0.0c	< 2.5±0.1d	7.4±0.1b
	Finished	7.6±0d	7.7±0.2c	6.5±0.2d	4.9±0.1c	4.3±0c	7.3±0bc

[a] Bacterial population was calculated to log CFU/g based on dry weight of compost.

[b] Data were expressed as means ± standard deviations of triplicates samples. Means with the different lower letters in the same column are significantly different ($P < 0.05$).

[c] Detection limit for direct plating count was calculated based on the moisture content of each compost sample.

.

Table 4.3 Population change of *L. monocytogenes* in biological soil amendments after 72 h incubation at room temperature

Farms	Compost type	Population change of *L. monocytogenes* (log CFU/g) in the compost with different MC	
		40% MC	80% MC
Dairy farm #1	Active	0.1±0	0.7±0.2
	Finished	0.1±0.2	0.5±0.4
Dairy farm #2	Active	-0.3±0.3	-0.4±0.2
	Finished	-0.6±0.2	-0.7±0.1
Dairy farm #3	Active	0.3±0.1	-0.3±0.3
	Finished	-1.1±0.2	-0.8±0.2
Poultry farm #1	Active	-0.4±0.1	-0.1±0.2
	Finished	-0.5±0.2	-0.8±0.1
Poultry farm #2	Active	-0.1±0.3	0.5±0.1
	Finished	-0.6±0.3	1.5±0.2
Poultry farm #3	Active	-0.3±0.2	-0.4±0.1
	Finished	0.1±0	-0.8±0.3

Table 4.4 Wilcoxon signed-rank test for experimental variables that affects the alpha diversities [a]

Experimental variables	Chao_richness	Shannon
Listeria inoculation	0.612	0.8385
Moisture contents	**0.015***	**0.0034****
Incubation time	**0.000006*****	**0.000000576*****

[a] Paired test for incubation time, unpaired for other two factors; Significant level was indicated by Benjamini-Hochberg FDR adjusted *P*- value, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 4.5 Canonical correspondence analysis outputs for each farm

Farms	Experimental factors	F	*P*-values [a]	χ^2
Dairy farm #1	**Experimental moisture**	**4.8637**	**0.005****	**0.16077**
	Incubation time	**6.2962**	**0.005****	**0.20812**
	L. monocytogenes inoculation	0.6487	0.917	0.02144
	Residual			1.45439
Dairy farm #2	Experimental moisture	1.1631	0.363	0.04667
	Incubation time	1.8146	0.169	0.07282
	L. monocytogenes inoculation	0.6486	0.917	0.02603
	Residual			1.76564
Dairy farm #3	**Experimental moisture**	**2.8826**	**0.033***	**0.11812**
	Incubation time	**2.9677**	**0.041***	**0.12161**
	L. monocytogenes inoculation	0.5889	0.917	0.02413
	Residual			1.80296
Poultry farm #1	Experimental moisture	1.2030	0.345	0.06119
	Incubation time	2.3772	0.113	0.12091
	L. monocytogenes inoculation	0.5597	0.917	0.02847
	Residual			2.23804
Poultry farm #2	**Experimental moisture**	**6.3641**	**0.005****	**0.23171**
	Incubation time	**9.1743**	**0.005****	**0.33403**
	L. monocytogenes inoculation	0.5080	0.917	0.01850
	Residual			1.60201
Poultry farm #3	Experimental moisture	1.5417	0.169	0.13613
	Incubation time	**2.7471**	**0.022***	**0.13613**
	L. monocytogenes inoculation	0.6603	0.917	0.03272
	Residual			2.18045

[a] Significant level was indicated by Benjamini-Hochberg FDR adjusted *P*- value, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 4.6 Relative proportions of selected functional categories found in active dairy compost samples that significantly changed among different treatments annotated by SEED subsystem level 1 and level 2 as revealed by metatranscriptomic sequencing

	Functional categories	No LM		With LM	
Level 1	Level 2	0h (%)	72h (%)	0h (%)	72h (%)
Amino Acids and Derivatives	Arginine; urea cycle, polyamines	8.02±0.02	9.65±0.34	7.85±0.12	**11.7±0.18**
Cell Wall and Capsule	Gram-Positive cell wall components	1.17±0.03	2.2±0.01	1.06±0.17	**3.3±0.11**
Clustering-based subsystems	Hypothetical Related to Dihydroorate Dehydrogenase	Low in proportions	Low in proportions	Low in proportions	Low in proportions
Clustering-based subsystems	Two related proteases	0.73±0.01	0.92±0.02	0.69±0.02	0.76±0.01
DNA Metabolism	DNA repair	62.35±0.05	61.11±0.4	62.98±0.16	56.84±0.12
DNA Metabolism	Unclassified	4.87±0.07	6.73±0	4.79±0.12	**9.07±0.44**
Phages, Prophages, Transposable elements, Plasmids	Phages, Prophages	17.69±2	48.57±3.2	15.11±0.18	**78.81±1.21**
Phages, Prophages, Transposable elements, Plasmids	Pathogenicity islands	78.97±2.1	48.21±3.97	82.23±0.52	17.76±0.6
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	79.55±0.94	84.09±0.35	87.89±0.24	**88.2±0.13**

[a] Significant at *P*-value < 0.001, and numbers highlighted in bold indicate a significant increase in the presence of *L. monocytogenes* after 72h.

Figure 4.1 Experimental design for 16S rRNA gene, metagenomic, and metatranscriptomic sequencing.

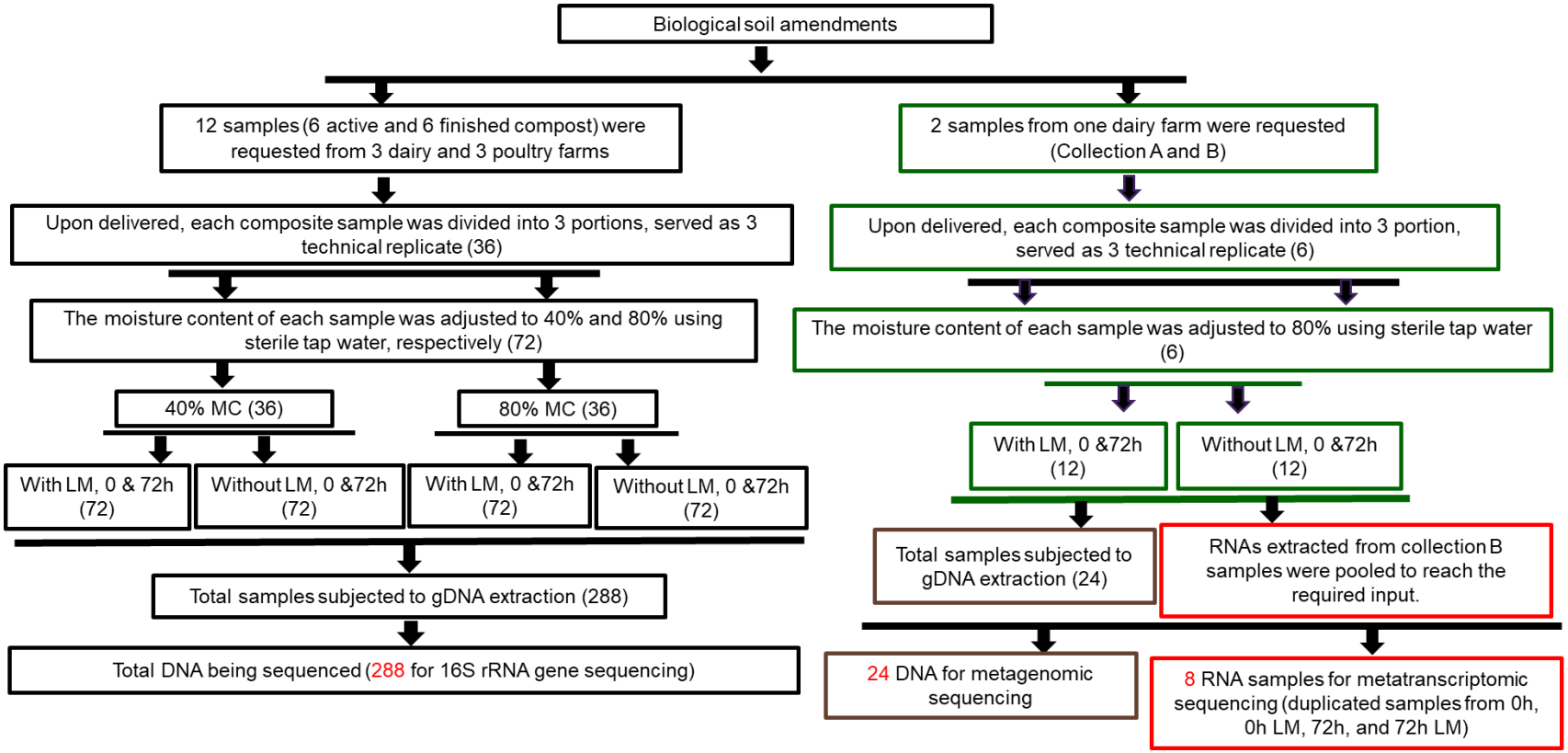


Figure 4.2 Relative abundance of the most common phyla (i.e., representing > 1% of total reads) present in four different compost types (dairy active compost, dairy finished compost, poultry active compost, and poultry finished compost) (A); LEfSe's output of differentially abundant bacterial taxa among four compost types (B). Significant bacterial genera were determined by Kruskal-Wallis test ($P < 0.05$) with LDA score greater than 2 (B).

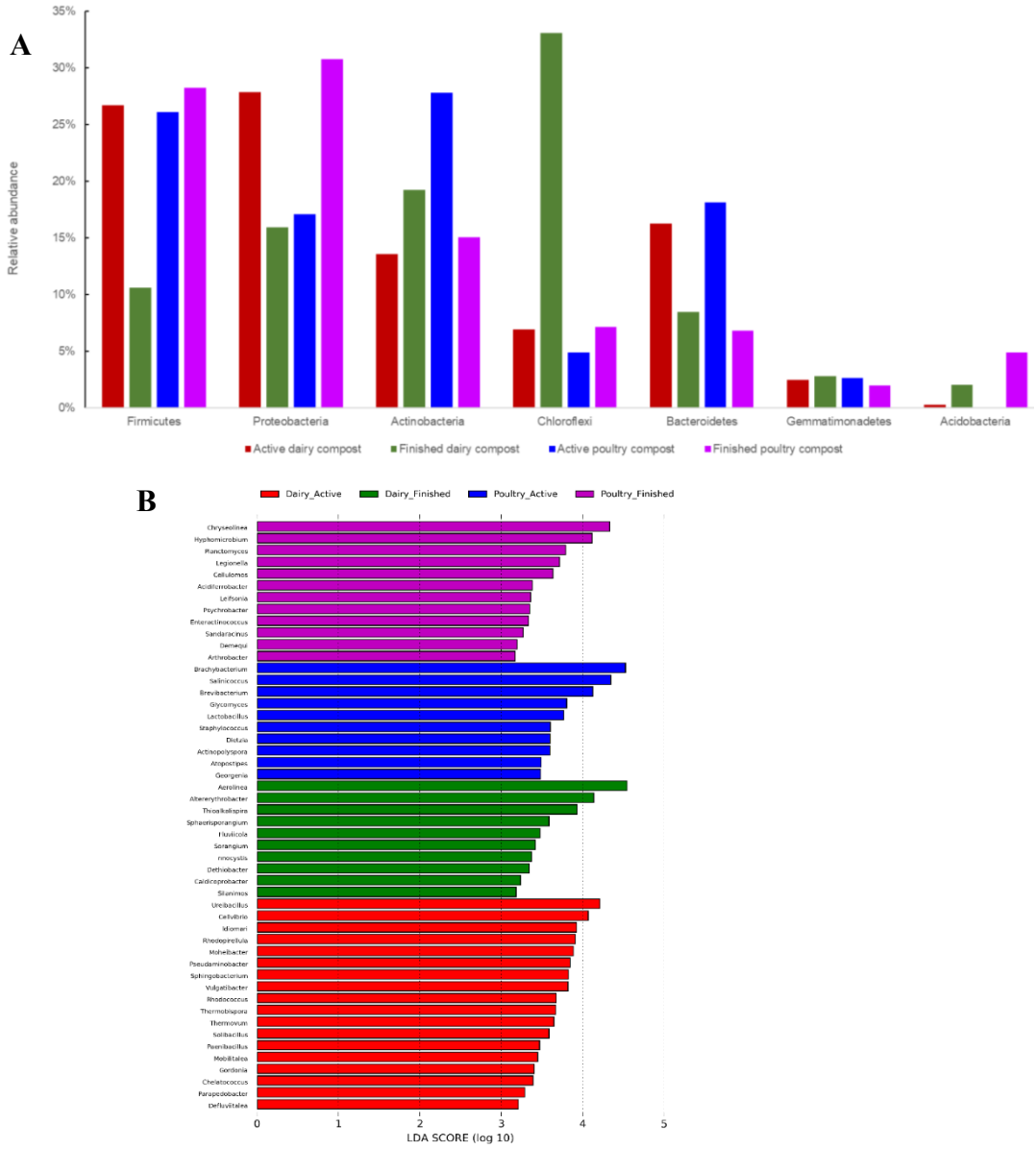


Figure 4.3 Bacterial community composition in dairy (A, B) and poultry (C, D) compost at phylum level, with X-axis represents different samples, and Y- axis stands for relative percentage of each bacterial phylum. Left 12 lanes for active compost samples, and right 12 lanes for the finished compost samples with different experimental treatments, respectively, in terms of *L. monocytogenes* inoculation (N for not inoculation, Y for with inoculation), moisture contents (40 and 80%), and incubation period of 0 h (A, C) and 72 h (B, D)

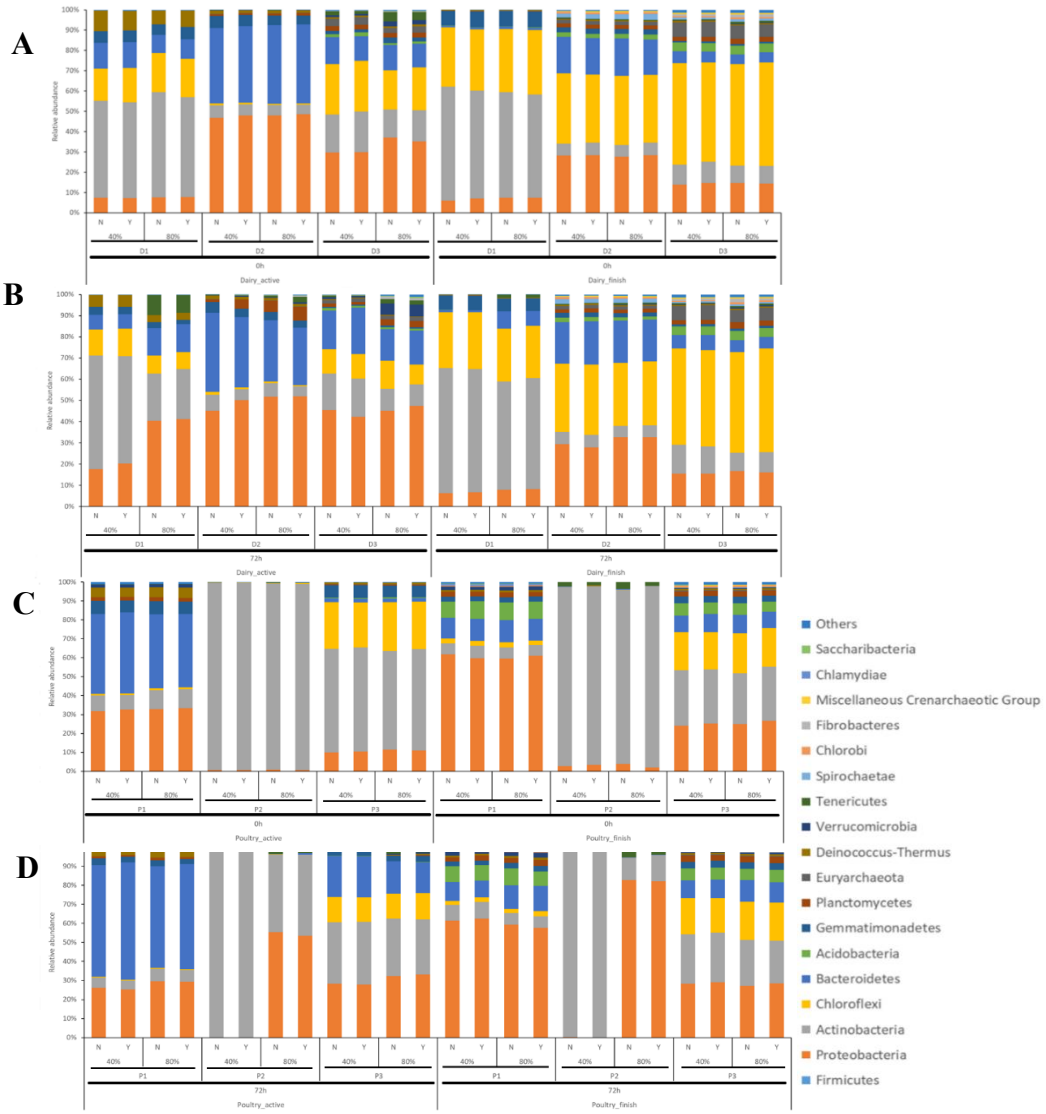


Figure 4.4 Effect of compost type on alpha diversity, chao richness (A) and Shannon_index (B), was tested using Kruskal-Wallis analysis, and the significant levels with Benjamini–Hochberg FDR adjusted *P*-value were added on the plots. The blue arrows indicate that the cultivated *L. monocytogenes* increased in these compost samples with 40 (light green) or 80% (green) moisture contents after 72 h of incubation. The effect from moisture contents on alpha diversity were tested using Wilcoxon signed-rank test, and the significant levels were added on the boxplot (ns; not significant difference with *P*-value > 0.05; *; significant difference with *P*-value < 0.05).

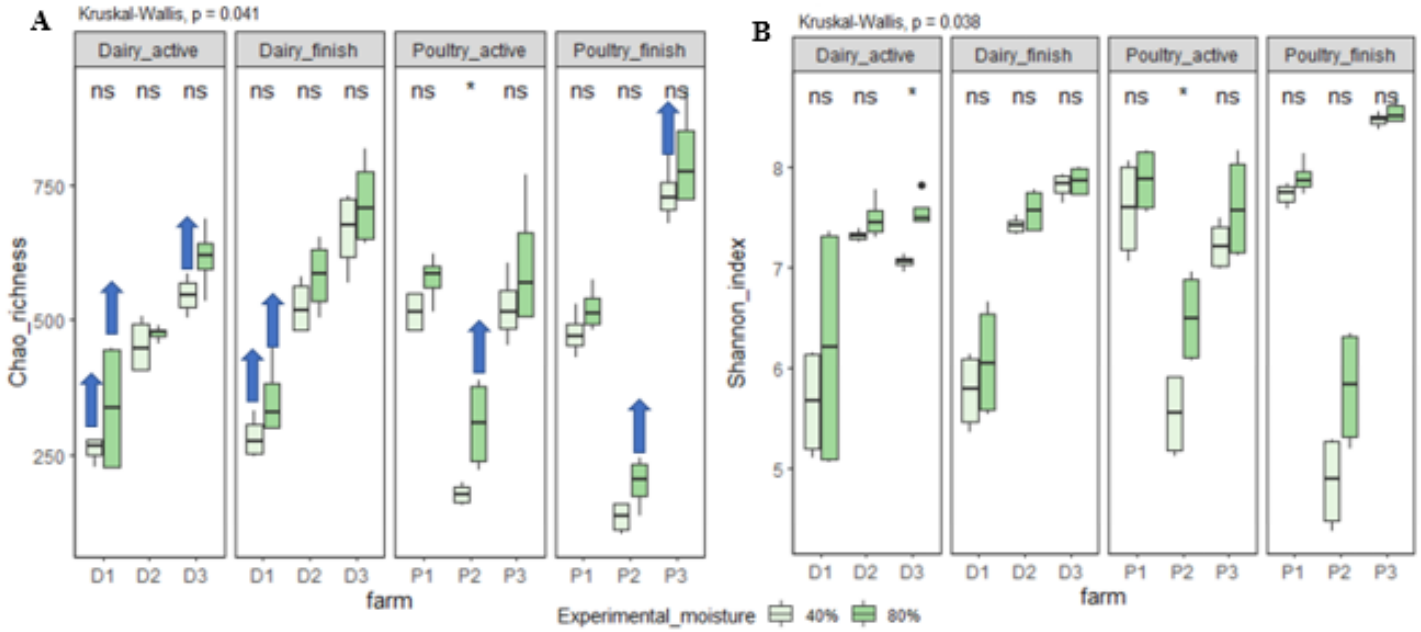


Figure 4.5 Weighted UniFrac distance-based principal-coordinate analysis (PCoA) plots for dairy (A, B, C, D) and poultry compost (E, F, G, H). Shaded polygons were applied to compost samples collected from the same stage (A and E), transparent polygons were applied to the compost samples with different treatments, including *L. monocytogenes* inoculation (B and F); experimental moisture contents (C and G), and incubation time (D and H).

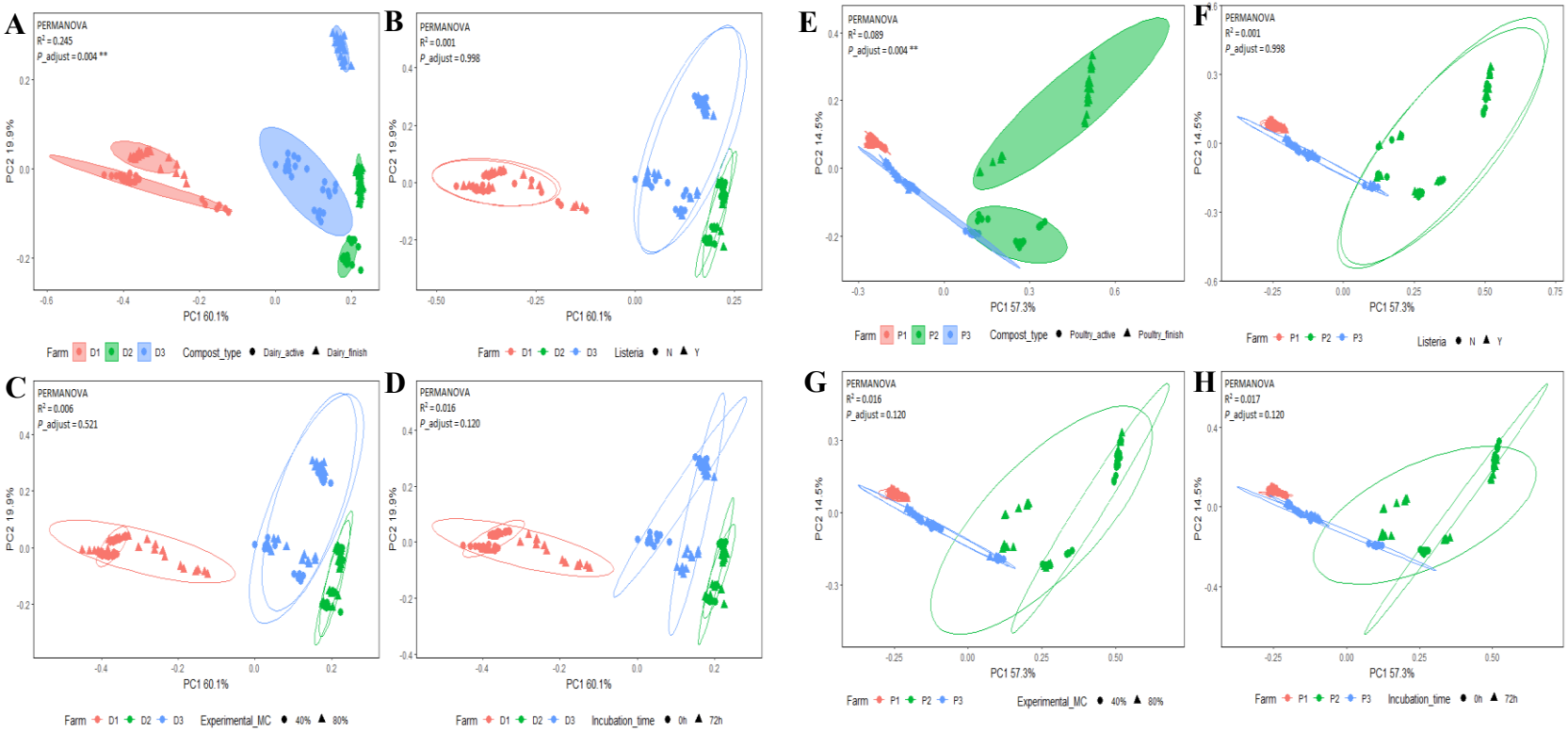


Figure 4.6 Similarities percentage (SIMPER) analysis screening top taxa driving variation in community composition due to the presence of *L. monocytogenes* in dairy (A) and poultry (B) compost samples, at 0 and 72 h incubation periods. The size of circles is indicating the percentages of decrease (blue) or increase (orange) in relative abundance of microbial member.

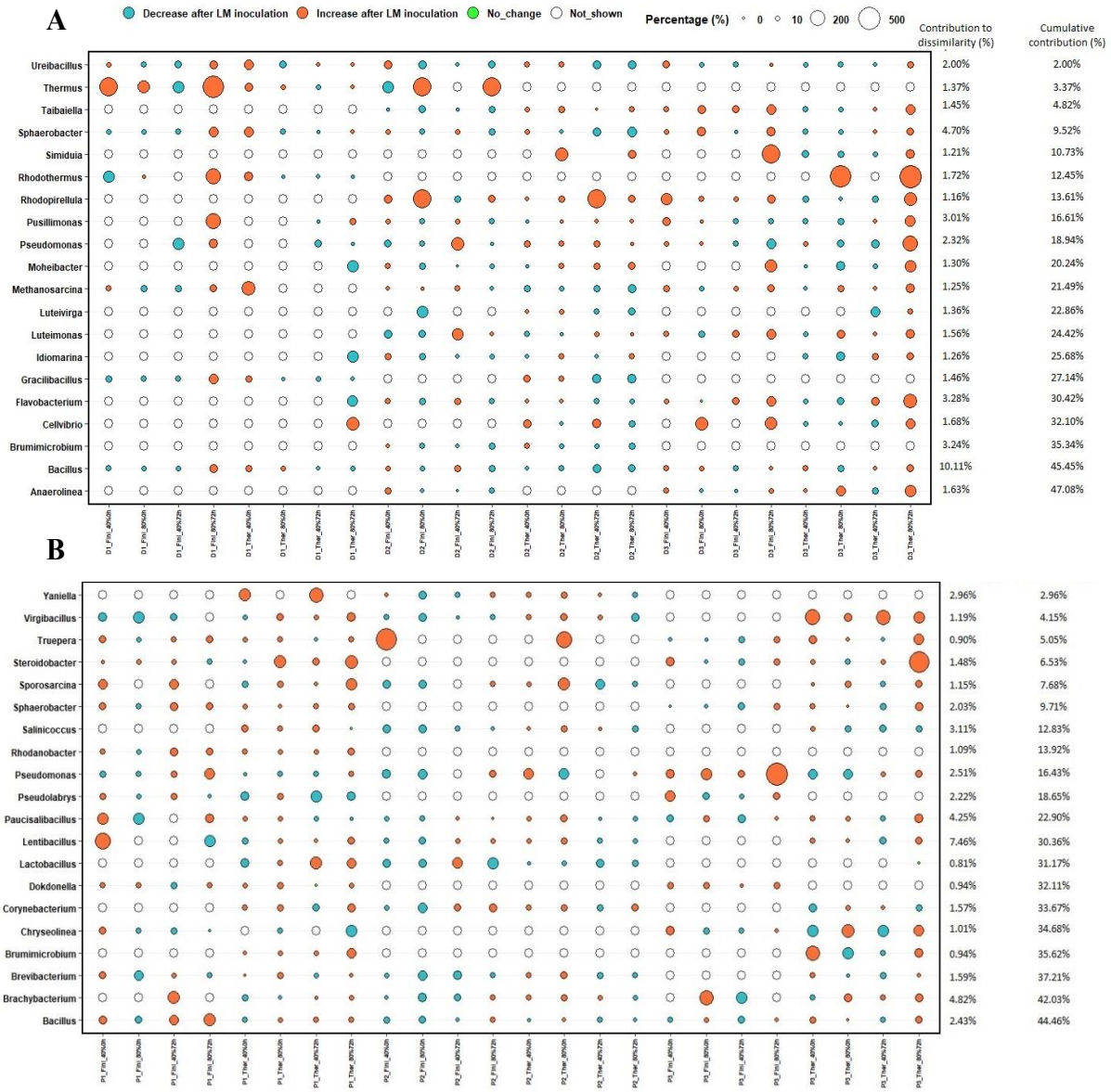


Figure 4.7 The relative abundance of discriminant microorganisms in compost samples collected from different farms.

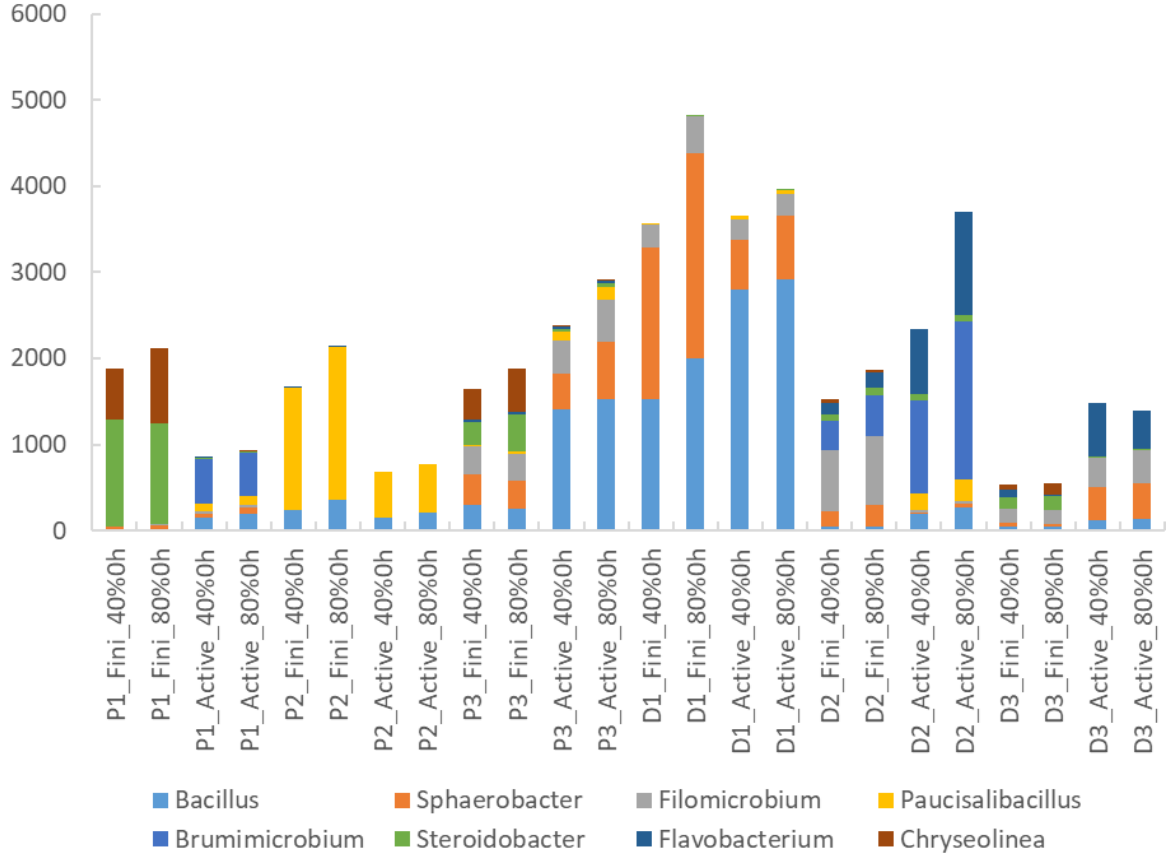


Figure 4.8 Functional profiles detected in active dairy compost microbiome annotated with SEED subsystem (level 1).

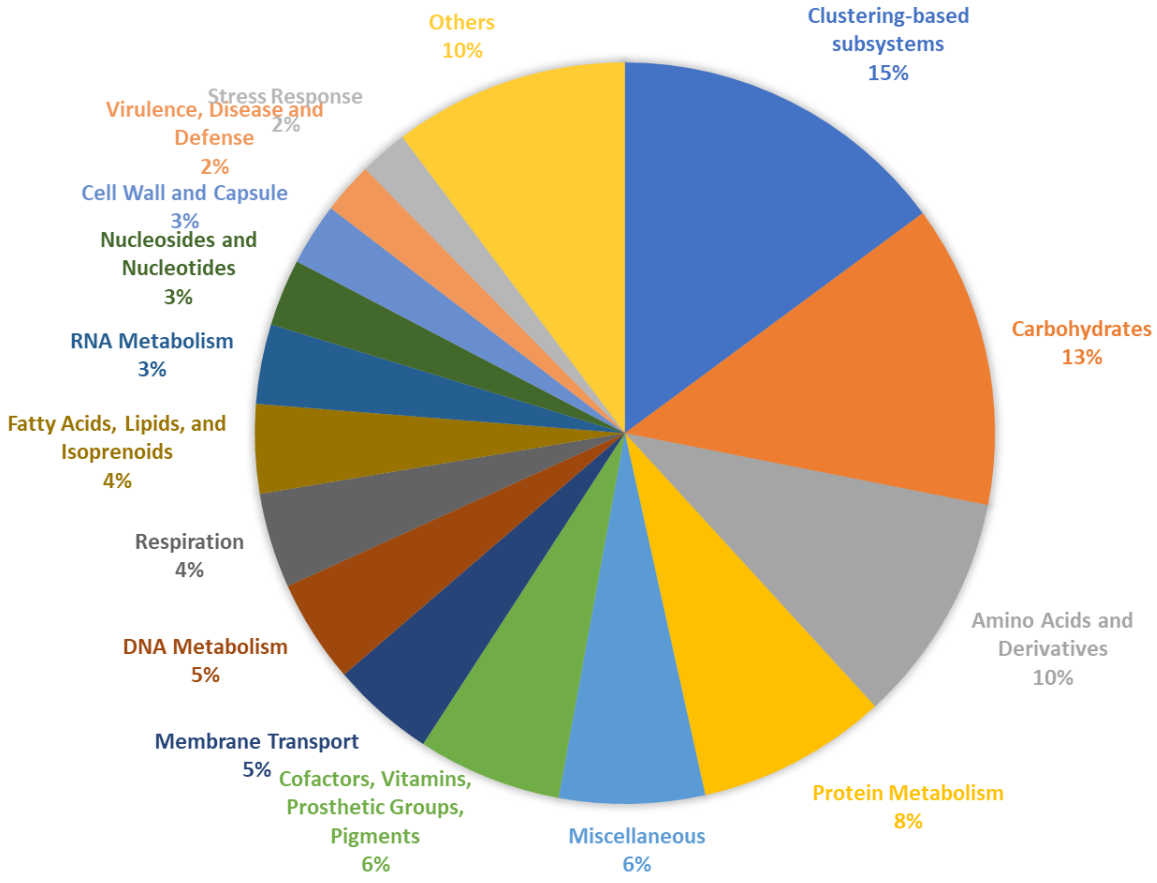


Figure 4.9 Functional profiles for the microbial metagenomes of active dairy compost from two separate collections (A and B). Extended error bar plot compared the functional profiles for the microbial metagenomes in active dairy compost from two separate collections based on the SEED subsystem level 1. Points and bars indicate the differences between collections A and B (blue and red, respectively), and the values at the right show the *P*-values were derived from a White's non-parametric t-test with Benjamini–Hochberg FDR correction.

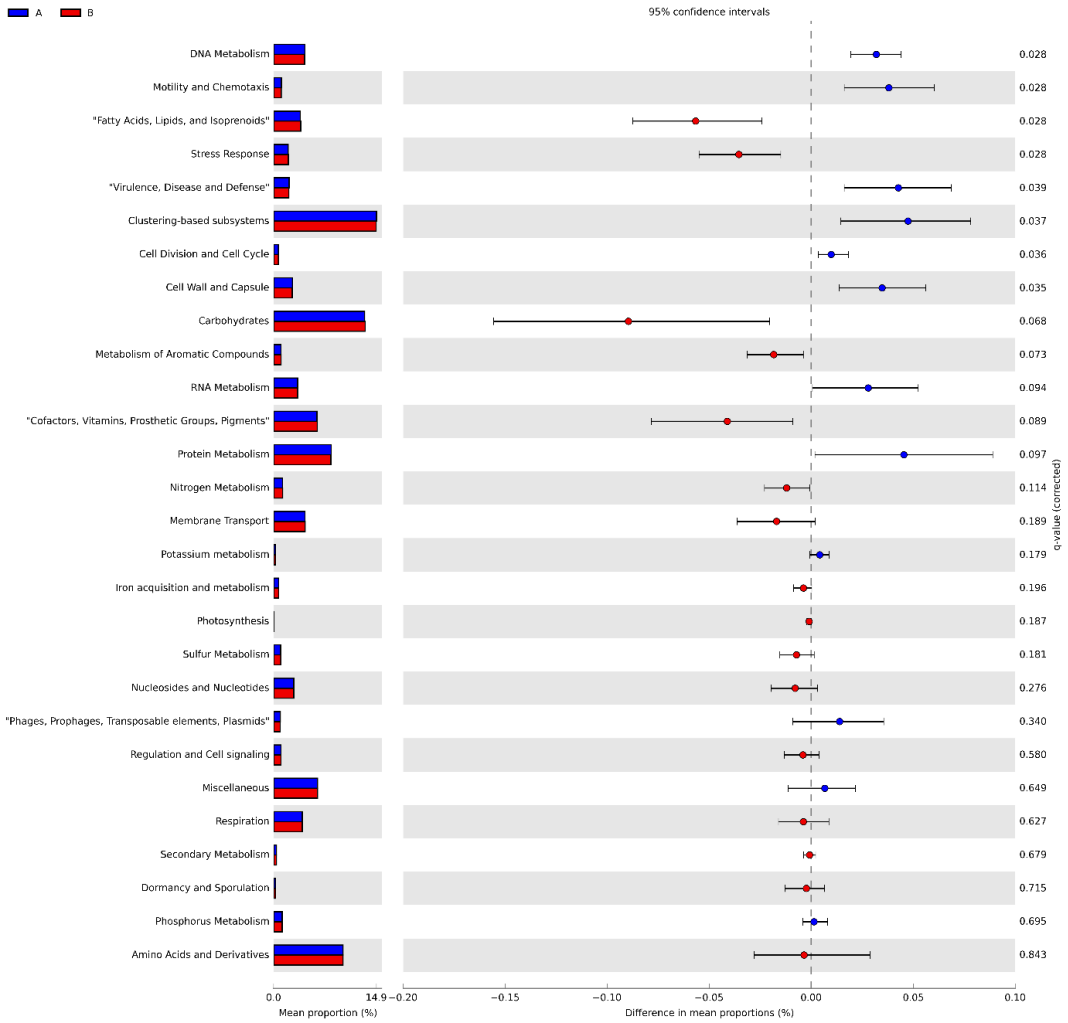


Figure 4.10 Extended error bar plot indicating the microbial functional potentials changed in active dairy compost from collection B due to the inoculation of *L. monocytogenes* after 72 h incubation based on the SEED subsystem function genes entries. Points and bars indicate the differences between *L. monocytogenes* inoculated- and uninoculated-compost (light blue and light orange, respectively), and the values at the right show the *P*-values were derived from a White's non-parametric t-test with Benjamini–Hochberg FDR correction.

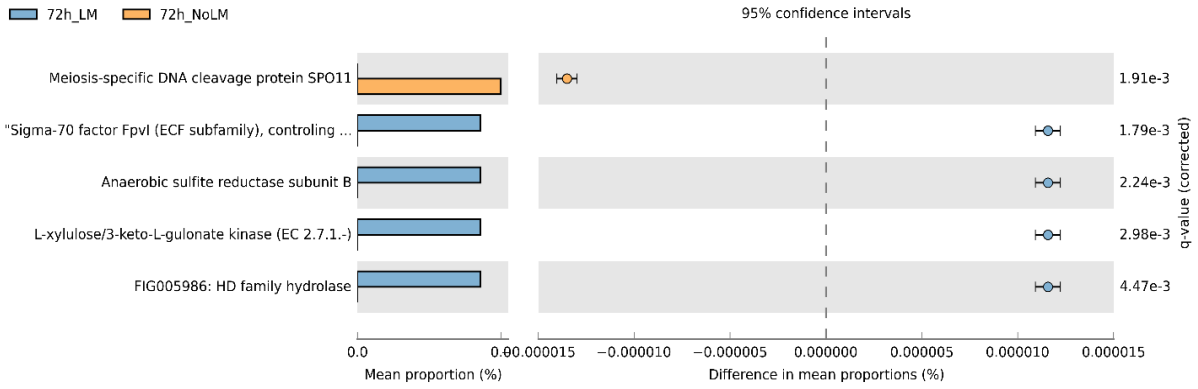
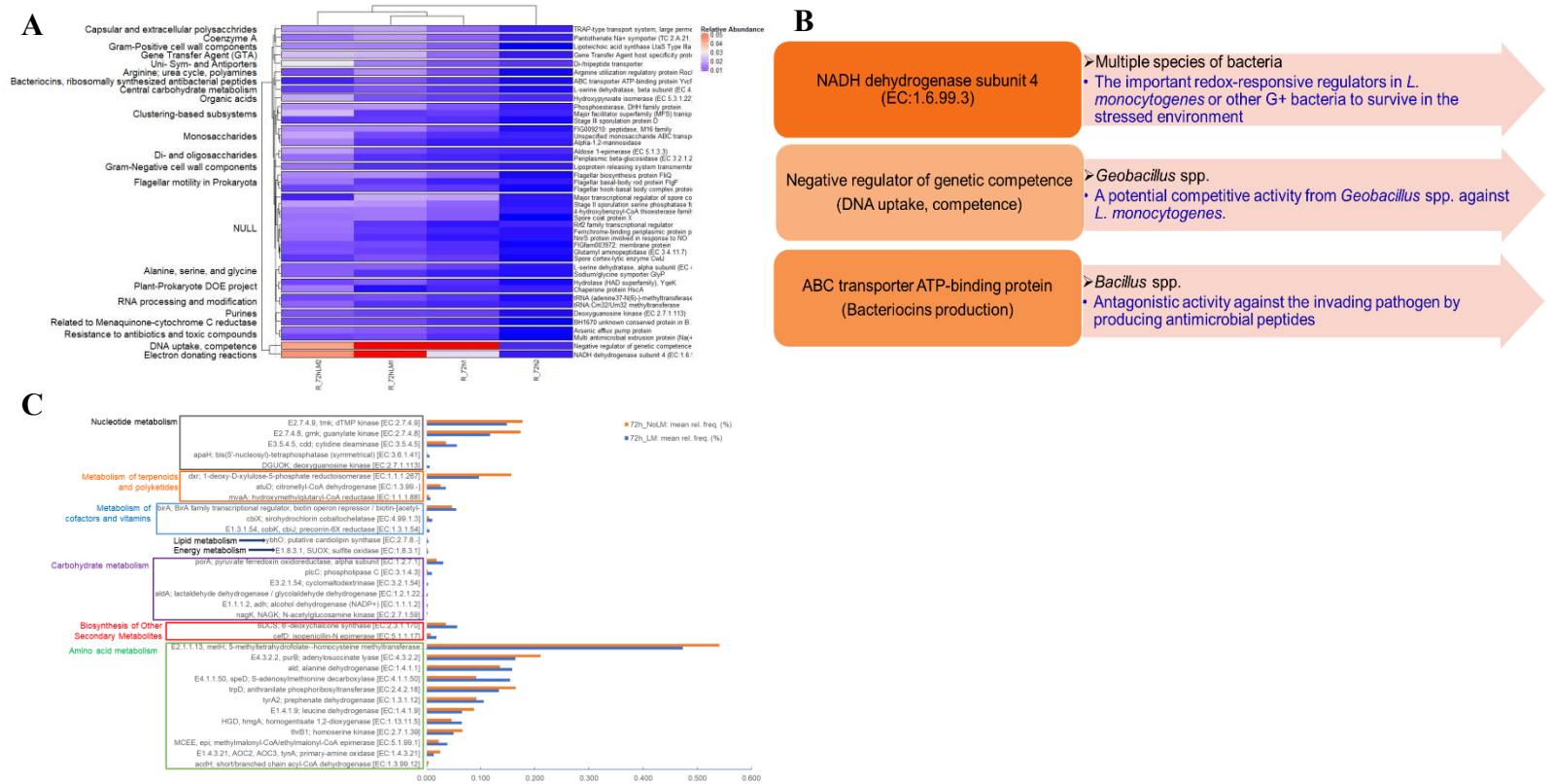


Figure 4.11 Genetic profile of the 43 significantly differentially abundant microbial genes expression between *L. monocytogenes* inoculated and uninoculated group as shown in heatmap (A), the gene list was clustered to hierarchy SEED subsystem level 2 as well (left column, A), with the gene induced in specific bacterial species (B), and changes in gene expression associated with selected microbial metabolisms in compost with and without the inoculation of *L. monocytogenes* as determined using KEEG database (C).



CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF COMPETITIVE EXCLUSION MICROORGANISMS FROM ANIMAL WASTE-BASED COMPOST

ABSTRACT

Competitive exclusion (CE) microorganisms have shown great potentials as environmental-friendly tools to control harmful microorganisms. Considering of dairy and poultry composts containing a diversity of microbial species, we hypothesized that the compost may be a good source for isolating compost-borne CE microorganisms, which can inhibit the growth of *Listeria monocytogenes*. In this study, CE strains were screened and isolated from composts using double- or triple-agar-layer methods. The addition of resuscitation promoting factor (Rpf) produced by *Micrococcus luteus* promoted the growth of slow-growing/viable but non-culturable species from composts. A total of 40 bacterial isolates were confirmed with anti-*L. monocytogenes* activities, and then tested for Gram-reaction, motility, biofilm-forming ability, and inhibitory spectra against produce outbreak-associated *L. monocytogenes* strains, followed by identification by 16S rRNA gene sequencing. About 50% of the isolated CE strains were identified as *Bacillus* spp., and 17 of 40 isolates can inhibit more than 10 produce outbreak-associated *L. monocytogenes* strains, while 9 CE strains isolated from poultry litter compost were confirmed as motile and competitive biofilm formers. Those 40 CE isolates based on the origin of isolation were separated into two groups, i.e. poultry and dairy CE groups, and then tested for anti-*L. monocytogenes* activity in both compost extracts and compost. After 168 h incubation with CE strains in compost extracts under all conditions, the growth potentials of *L. monocytogenes* were reduced by co-culturing by 0.1- to 1.9-log depending on incubation

temperature, types, and ratio of the compost extracts. Results showed that the inhibition effect from CE strains was higher in more concentrated compost extract (1:5) at 35°C. In compost samples, the addition of CE strains reduced *L. monocytogenes* population by ca. 1.2 log CFU/g at room temperature after 24 to 168 h incubation. The efficacy of CE strains against *L. monocytogenes* was stronger in the dairy compost as compared to that in the poultry litter compost. Findings from this study suggested that compost-adapted CE microorganisms have the potentials as a biological control agent to control *L. monocytogenes* in agricultural environments.

INTRODUCTION

Listeria monocytogenes is widespread in the environment and can survive well in sewage-sludge, plants and decaying vegetation, soil, animal hives, and feces (Weis and Seeliger, 1975). In the U.S., *L. monocytogenes* is responsible for 19% of the total deaths due to the consumption of contaminated food, with fresh produce as an important source of contamination at both farm and processing environments (Scallan et al., 2011). Oliveira et al. (2011) reported that *L. monocytogenes* was transferred from soil amended with contaminated organic compost to lettuce leaves, indicating that contaminated biological soil amendments can transmit this pathogen to fresh produce.

One of the most environmental-friendly methods available to control pathogens is the use of competitive exclusion (CE) microorganisms. CE microorganism also has been described as “bacterial antagonism” or “bacterial interference”, which is based on the involvement of non-pathogenic microorganisms to enhance the microbial competition in

order to reduce pathogens in a certain environment (Nurmi et al., 1992). Traditionally, lactic acid bacteria and their associated commercial products have been well-documented for their antimicrobial activities against the growth of *Salmonella*, *L. monocytogenes*, and other pathogens (Muñoz et al., 2019; Gensler et al., 2020). For example, Zhao et al. (2006) successfully used two lactic acid bacteria to inhibit *L. monocytogenes* in floor drains of a poultry processing plant at 3 to 26°C. But, the wide application of these lactic acid bacteria in agriculture fields has been limited due to the high cost and special growth requirement of lactic acid bacteria. Thereby, more cost-effective applications of CE for pathogen control in agriculture field need further study.

As microbial diversity is a key factor in avoiding pathogen outbreaks, it becomes urgent to search for effective CE microorganisms from environmental sources where *Listeria* reside and adapt. According to Jiang et al. (2009), indigenous microorganisms in compost can possess the suppressive activities against foodborne pathogens. Kim and Jiang (2010) showed that the regrowth of *Salmonella*, *Escherichia coli* O157:H7, and *L. monocytogenes* in dairy compost or physically heat-treated poultry litter occurred only when there was a lack of competitive indigenous microorganisms. As indicated from these studies, bacterial competition in the complex compost ecosystem is surely expected. Some microorganisms likely possess competitive features such as the secretion of biocidal compounds and fast growth rate. Therefore, further investigation on isolating CE microorganisms from compost is needed for pathogen control.

Culture-based methods are essential for isolating microorganisms. Due to the presence of a significant large population of non-culturable microorganisms in the natural

environment and our limited knowledge on indigenous microbial species, only less than 1% of the microorganisms present in the environment have been cultivated with currently culture-based methods (Ramamurthy et al., 2014). Recent attempts such as adding specific nutrients and substrates or resuscitation promoting factors, supernatants of growing cells, and modifying incubation conditions have been investigated to resuscitate or recover those not previously cultivated microorganisms (Pinto et al., 2011; Chen et al., 2012). For example, Jin et al. (2017) successfully isolated viable but non-culturable (VBNC) bacteria from printing and dyeing waste-water bioreactor with the culture media containing resuscitation promoting factor (Rpf) protein secreted by *Micrococcus luteus*. Moreover, it is not surprising that CE microorganism can be a slower grower, hard to culture, or even enter VBNC stage in compost (Su et al., 2018). However, to date, no such efforts have been attempted to isolate CE microorganisms from animal wastes or other soil amendments, albeit there are very limited studies documenting the use of CE microorganisms as a biological control against to reduce *L. monocytogenes* in compost.

Therefore, the objectives of this study were to isolate compost-borne CE microorganisms with anti-*L. monocytogenes* activities and then verify antagonistic activities of those CE cultures against *L. monocytogenes* in both compost extract and compost models. Ultimately, these compost-borne CE microorganisms isolated from this study with anti-*Listeria* activity could be used to control *L. monocytogenes* contamination in biological soil amendments to ensure the safe production of fresh produce.

MATERIALS AND METHODS

Compost sample collection. A total of 12 compost samples (6 dairy and 6 poultry wastes–based composts) were collected from six different facilities located in multiple states in the US, including Arizona, California, Michigan, South Carolina (n=2), and Wisconsin. From each facility, samples of both active compost (collected from the thermophilic composting stage [> 55°C /131°F] within 1 month of composting) and finished compost (3 to 6 months of composting), with the same ingredients were requested. In accordance with the sampling protocol recommended by the California Leafy Greens Marketing Agreement (LGMA, 2010), the composite compost samples were collected in Ziploc bags, shipped under the ambient conditions to our lab, and stored at refrigeration conditions (4°C) once received.

Preparation of *L. monocytogenes* cultures. *L. monocytogenes* strains, including 101M, LCDC 81-861, and Scott A, were obtained from the culture collection by Dr. Mike Doyle at the Center for Food Safety, University of Georgia. Other produce outbreak-associated *L. monocytogenes* strains (FSL R2-0503, J1-0107, J1-0101, R9-0506, R9-5507, J1-0031, J1-0158, S10-2161, and R9-5506) and one surrogate strain *L. innocua* FSL-C2-0008 (Table 5.1) were kindly provided by Dr. Martin Wiedmann at Cornell University. *L. monocytogenes* strains, including 101M, LCDC 81-861 and Scott A, all serotype 4b, were used for screening CE candidate strains from compost samples. Besides, produce outbreak-associated *L. monocytogenes* strains and the surrogate strain, *L. innocua*, were also tested for the anti-*Listeria* spectrum of candidate CE strains and biofilm-forming abilities. Each strain was individually grown in tryptic soy broth with 0.6% yeast extract (TSBYE; Becton

Dickinson, Sparks, MD) at 37°C for 16 h. The cultures were collected by centrifugation at 3,000x g for 15 min and resuspended in sterile saline (0.85%, w/v NaCl). The optical density at 600 nm of each strain was adjusted with sterile saline to the desired concentration.

Screening potential CE strains from compost for anti-*L. monocytogenes* activities using the double-agar-layer method. Compost samples collected from different farms were used for the initial screening of CE candidate strains by the double-agar-layer method. Briefly, serial dilutions of the compost samples were made with sterile saline, and the proper dilutions were plated onto tryptic soy agar (TSA; Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 h. Following incubation, the plates with less than 30 colonies were overlaid with a second layer of 2% (w/v) TSA soft agar spiked with ca.10^7 CFU/ml of *L. monocytogenes* strains 101M, LCDC 81-861, or Scott A, respectively. After incubation, colonies with the growth inhibition zone were picked up using a pre-sterilized tooth stick to break the top layer of soft agar, followed by further purification on TSA plates with several transfers. The total numbers of colonies picked up from the original plates depended on the observation of inhibition zones.

Screening potential CE strains from compost for anti-*L. monocytogenes* activities by the modified triple-agar-layer method. Two types of modified agar plates, i.e. most probable number (MPN) agar + resuscitation promoting factor (Rpf) (Jin et al., 2017) and proteose-yeast-glucose (PYG) agar (Kato et al., 2018), were used in an effort to grow the viable but hard-to-culture bacteria from active compost samples. The MPN medium consisted of 0.05% (w/v) yeast extract, 0.5% (w/v) peptone, 0.25% (w/v) NaCl,

0.5% (w/v) glucose, 15% (w/v) agarose, and 10% prepared Rpf (MPN + Rpf). Briefly, *Micrococcus luteus* ATCC 4698 was used to prepare Rpf. *M. luteus* was inoculated to the lactate minimal medium (LMM; containing 5 g peptone, 3 g yeast extract, and 1 g $MgSO_4$•7H2O, pH 7.0 per liter). Based on procedures described by Jin et al. (2017), the fermentation broth containing Rpf was prepared, centrifuged, and then filtered through a 0.22 μm filter (VWR International, Radnor, PA) to remove cells. Finally, the supernatant containing Rpf was obtained, stored at −20°C, and used as supplemental material for MPN + Rpf agar media preparation. Besides, in order to improve the cultivability of previously uncultured slow-growing bacteria, PYG agar was prepared by autoclaving the phosphate and agar separately according to Kato et al. (2018).

Serial dilutions of the compost samples were made with sterile saline, and the proper dilutions were plated onto PYG and MPN + Rpf agar plates. The second layer of agar (2%, w/v) was supplemented with nystatin (100 μg/ml) to prevent fungal growth during the extended incubation time (Powell et al., 2007). PYG and MPN + Rpf plates were incubated at 25°C in the dark for 3 weeks, and at 25°C for 7 days, respectively. For PYG agar plates, new colonies that appeared after 7 days of incubation were marked on days 14 and 21. Following incubation, colonies on both PYG and MPN + Rpf agar were then overlaid with a third layer of 2% (w/v) TSA soft agar spiked with ca. 10^7 CFU/ml of *L. monocytogenes* strains 101M, LCDC 81-861 or Scott A, respectively. After incubation for 24 h at 37°C, colonies with inhibition zone were picked up as described above, followed by transferring to fresh TSA for purification.

Confirmation and characterization of CE isolates and metabolites. The antagonistic activity against *L. monocytogenes* by the CE candidate strains was further confirmed by the spot-on-lawn method (Zhao et al., 2006). Briefly, the CE isolates were spot-inoculated onto the TSA plates. After overnight incubation at 37°C, the colonies on the plates were first treated by exposure to chloroform vapor for 15 min and then overlaid by different *L. monocytogenes* strains in soft agar (2%, w/v), respectively. The zone of inhibition was observed after overnight incubation at 35°C.

All the candidate CE strains confirmed by spot-on-lawn method were tested for anti-*Listeria* activities against those produce outbreak-associated *L. monocytogenes* and surrogate strains by both spot-on-lawn for colonies, and agar-well diffusion methods were for testing metabolites from CE isolates. The positive and negative isolates were isolated in this work and confirmed to produce or not to produce extracellular antimicrobials to *L. monocytogenes*, respectively. To prepare for the cell-free supernatant for the agar-well diffusion method, individual CE strain was grown overnight at 35°C for 18 h, and then transferred twice in brain heart infusion broth (BHI, Becton Dickinson, Sparks, MD) to accumulate more metabolic substances in the stationary stage. After 36 h of incubation at 35°C, the cell-free supernatant containing the metabolic substances of these enrichment cultures were collected by filter sterilization using a 0.22 μm filter (VWR International, Radnor, PA). Then, the thick TSA plates (ca. 1 – 1.2 cm in depth) with *Listeria* lawn were prepared by spread-plating ca. 10^7 CFU/ml of individually tested *Listeria* strain using a sterilized cotton swab for an even distribution of inoculum. The cylinders of 6.0 mm diameter were cut and removed by a sterilized glass tube with a sharp edge from these pre-

inoculated TSA agar plates. Next, 25 µl of soft agar were deposited to each well bottom, in order to seal the well bottom. About 100 µl of cell-free supernatant were added into each well. The zone of inhibition was observed after overnight incubation at 37°C.

The candidate CE strains were also characterized by Gram-staining followed by the motility test using wet mount or sulfide-indole-motility (SIM) medium (Shields et al., 2011). Moreover, as a potential application of CE strains for the composting process, all the candidate CE strains were tested for the ability of growth at 55°C.

Identification of candidate CE strains by Sanger sequencing. Primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3’) were used for amplifying 16S rRNA gene (Kim et al., 2011). DNAs from CE isolates were extracted by Promega Wizard® genomic DNA purification kit (Promega, Madison, WI). The final reaction mixture included 20 ng of template DNA, 2.5 µl 10X PCR buffer, 50 mM $MgCl_2$, 20 mM dNTP’s, 10 µM of each primer, and 1 U of Taq DNA polymerase in a final reaction volume of 25 µl. Initial DNA denaturation was performed at 96°C for 2 min in a Mastercycler ep® gradient S thermal cycler (Eppendorf, Hamburg, Germany), followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 30 s, and then elongation at 68°C for 1.5 min, which was followed by a final elongation at 72°C for 10 min. The amplified PCR products were purified with the Wizard® genomic DNA purification kit (Promega, Madison, WI). Sequencing reactions of the amplified PCR products were performed by Eurofins Genomics (Louisville, KY). Sequences obtained were blasted with the GenBank database [http://www.ncbi.nlm.nih.gov/. 16S Ribosomal

RNA Sequences (Bacteria and Archaea)] for species or genus assignment. The highest identity (> 95%) was selected as the identified species or genus.

Biofilm-forming abilities of produce-associated *L. monocytogenes* strains and CE isolates. Each *L. monocytogenes* and candidate CE strain was cultured separately in a 15-ml centrifuge tube containing TSBYE broth at 37°C. After incubation, each overnight culture was diluted to ca. 10^4 CFU/ml in TSBYE. Next, 20 µl of diluted culture were transferred to 190 µl of fresh TSBYE medium in a 96-well plate using four wells per strain and incubated at 37°C for 3 days. Sterile TSBYE was used as the negative control. To avoid the "edge effect" primarily caused by evaporation during incubation, 200 µl of autoclaved distilled water was added in peripheral wells to reduce the water loss as suggested by Shukla et al. (2017). After 3 days of incubation, biofilm formation was quantified using crystal violet at $OD_{570\ nm}$ according to the method described by O'Toole et al. (2011). *L. monocytogenes* strain with the strongest biofilm formation was selected as the control for comparing with the biofilm-forming ability with each candidate CE strain. A standard was then made for these candidate CE strains after compared with *L. monocytogenes* control, i.e. $OD_{570\ nm}$ (CE) $\leq$ $OD_{570\ nm}$ (control) classified as non-competitive biofilm former, whereas $OD_{570\ nm}$ (CE) > $OD_{570\ nm}$ (control) classified as competitive biofilm former (Stepanović et al., 2000).

Application of CE microorganisms to inhibit the growth of *L. monocytogenes* in compost extracts and commercial composts. Two types of *L. monocytogenes*-free compost, including finished poultry and dairy composts, were used for inhibition test directly or preparing compost extracts first, and then used for growth inhibition study. For

compost extract preparation, each compost was extracted with sterile tap water at two ratios (1:5 and 1:10, w/v) in a shaking incubator (100 rpm), at 22°C for 24 h, followed by centrifugation at 5,000 x g for 20 min. The supernatant was filtered through a 0.22 μm filter, stored at -20°C until used. Next, candidate CE strains originally isolated from poultry and dairy composts were tested in poultry and dairy compost extracts or compost samples, accordingly (Figure 5.1). The compost extract or compost samples with a cocktail of the rifampicin-resistant *L. monocytogenes* strains (R9-5506, Scott A, and 101M) (ca. 3- or 5-log CFU/ml or g in compost extract or compost sample, respectively) was inoculated with or without CE strains (ca. 7 log CFU/ml or g), and then incubated for up to 7 days. The inhibition study in compost extracts was performed at both 37°C and room temperature, whereas the inhibition study in solid compost was only performed at room temperature. At selected intervals (0, 1, 3, 5, and 7 days), the samples were serially diluted, and the proper dilutions were plated onto TSA plates supplemented with rifampicin (100 μg/ml) for enumeration of *L. monocytogenes* (Figure 5.1).

Statistical analysis. Data analysis was performed using JMP 11.2.1 (SAS Institute Inc., Atlanta, GA). Analysis of variance, followed by the least significant differences test, was carried out to determine whether significant differences ($P < 0.05$) existed among different treatments.

RESULTS

Isolation and characterization of CE strains from compost samples. No significant differences were found in the population levels of total compost bacteria as

enumerated by MPN + Rpf or TSA agar plates (Data not shown). However, colonies with different morphologies were observed on both PYG and MPN + Rpf as compared to the colonies grown on the regular TSA plates. After 14 and 21 days of incubation, the number of new colonies on PYG agar plates ranged from 8.4 to 156.3%, and 3.7 to 43.8%, respectively, as compared to the number of colonies that appeared on PYG agar plates from the highest countable dilution on Day 7 (Table 5.2). However, none of isolates grown on PYG agar plates (on day 7, 14, and 21 days) showed the anti-listeria activity. A total of 40 isolates (15 from poultry compost and 25 from dairy compost) were isolated from either TSA or MPN + Rpf plates and confirmed with anti-*L. monocytogenes* activities with the zone of inhibition ranging from 0.8- to 12-mm diameter (Figures 5.2 A and B).

Characterization and inhibitory spectra of candidate CE strains. The candidate CE strains were further characterized. Of these 40 CE isolates, 38 CE isolates were G+ (23 rods and 15 cocci), 2 isolates were identified as Gram-variable rods, 19 isolates were motile, 13 isolates grew at 55°C, whereas 3 of these 13 isolates were identified as having weak growth (Table 5.3).

Next, all isolates from the initial screening were evaluated individually for their abilities to inhibit the growth of produce outbreak-associated *Listeria* strains. As a result, 17 of 40 isolates can inhibit the growth of more than 10 *L. monocytogenes* or surrogate strains. However, only 5 of 17 isolates showed the inhibition to selected *L. monocytogenes* when cell-free supernatants were tested (Figure 5.2 C). Further, all CE candidate strains were identified by 16S rRNA gene sequencing, and these CE isolates were confirmed as

Bacillus spp. (n=20), *Kocuria* spp. (n=12), *Paenibacillus* spp. (n=4), *Brevibacillus* spp. (n=1), and *Planococcus* spp. (n=3).

Biofilm-forming abilities of candidate CE strains. *L. monocytogenes* is well-documented in forming biofilm by self or with other species in the environment (Van et al., 2010). To evaluate the biofilm-forming abilities of CE isolates, we first selected a control strain with the strongest biofilm-forming ability from produce outbreak-associated *L. monocytogenes* strains and then evaluated the biofilm-forming abilities of CE isolates by comparing to this control strain. After 72 h of incubation, biofilm-forming ability varied significantly among different strains of *L. monocytogenes*, ranging from OD_{570nm} of 0.12 to 0.58 ($P < 0.05$; Figure 5.3 A). Based on the OD_{570nm} value, strain L9 (serotype 1/2a, denoted as lineage II), was selected as a positive control strain for further testing. As shown in Figure 5.3 B, candidate CE strains, including two isolates (D4 and D7) from dairy compost and 9 isolates (P3, P5-P10, P13, and P15) from poultry compost, formed significantly more biofilm ($P < 0.05$) than *L. monocytogenes* control strain did as indicated by the higher absorbance values, therefore were designated as competitive biofilm formers. Taking consideration of mobility and strain-identification results, above 9 CE strains isolated from poultry litter compost, which belong to *Bacillus* spp., *Paenibacillus* spp., or *Brevibacillus* spp., were characterized as motile and competitive biofilm formers as compared to L9 strain. However, dairy isolates (D4 and D7) were not motile.

Application of CE microorganisms to inhibit the growth of *L. monocytogenes* in compost extracts and solid compost samples. Water extracts of two types of compost, including finished poultry and dairy composts with two extraction ratios (1:5 and 1:10)

were used to evaluate the effect of temperature and nutrient availability on the efficacy of CE isolates against *L. monocytogenes*. During the growth inhibition study, CE populations increased by 0.3 to 0.6 log in the dairy compost extracts (Figure 5.4), but no change ($P > 0.05$) was observed for CE populations in poultry compost extracts (Figure 5.5). After 24 h incubation, the growth potentials of *L. monocytogenes* in dairy compost extracts with extraction ratios of 1:5 and 1:10 were reduced by 2.2 and 1.7 log at 35°C or 1.6 and 1.5 log at room temperature, respectively, whereas the growth potentials of *L. monocytogenes* in poultry compost extracts with extraction ratios of 1:5 and 1:10 were reduced by 0.7 and 0.5 log at 35°C or 0.2 and 0.3 log at room temperature, respectively. After 168 h incubation, the growth potentials of *L. monocytogenes* in dairy compost extracts with extraction ratios of 1:5 and 1:10 were reduced by 0.5 and 0.1 log at 35°C or 1.9 and 0.7 log at room temperature, respectively, whereas the growth potentials of *L. monocytogenes* in poultry compost extracts with extraction ratios of 1:5 and 1:10 were reduced by 1.1 and 1.0 log at 35°C or 0.9 and 0.7 log at room temperature, respectively. Clearly, the growth potentials of *L. monocytogenes* were reduced by co-culturing with CE strains in both dairy and poultry compost extracts at all incubation conditions, but the inhibition effect from CE strains was more pronounced ($P < 0.05$) in the more concentrated dairy compost extracts (1:5) at 35°C followed by room temperature, especially after 24 h of incubation.

In order to test the efficacy of CE strains on controlling *L. monocytogenes* in commercial compost during storage, the survival of *L. monocytogenes* inoculated in dairy or poultry wastes-based compost in the presence of CE strains was determined at room temperature. As shown in Figure 5.6, no significant change ($P > 0.05$) was observed for

the total bacterial population in both dairy and poultry composts. In dairy compost (Figure 5.6 A), the population of *L. monocytogenes* increased by 0.4 log CFU/g after 24 h in the absence of CE strains, however, no regrowth was detected for *L. monocytogenes* when co-culturing with ca. 7-log of CE strains. In poultry compost (Figure 5.6 B), the regrowth potential of *L. monocytogenes* was slightly ($P > 0.05$) reduced in the presence of CE strains after 24 h. Overall, after 168 h incubation, the addition of CE cultures reduced *L. monocytogenes* population by 1.3 and 1.1 log CFU/g in dairy and poultry compost, respectively, with the efficacy of CE strains was slightly stronger ($P > 0.05$) in the dairy compost as compared to that in the poultry compost.

To find out the impact of compost types on the growth of CE strains and L. *monocytogenes*, the growth curves were generated for both CE and *L. monocytogenes* strains in dairy or poultry compost extracts (1:5). As shown in Figure 5.7, the population of CE strains increased by ca. 2-log in both extracts, as compared to ca 1-log growth of *L. monocytogenes* in both extracts. Clearly, a faster growth rate was observed for CE strains as compared to that of *L. monocytogenes* under the same incubation conditions.

DISCUSSION

Animal waste-based compost is rich in microbial species, and the compost microbial members thereby live in highly competitive environment. Thus, our initial goal was to isolate CE strains native to the compost environment that would compete-well against *L. monocytogenes*. Some special stimuli such as adding growth promoting factor (Rpf) or modification of preparation protocol (PYG agar), with extended incubation period

(7 – 21 days) were attempted to isolate compost-borne CE strains. Significant numbers of new colonies were grown on PYG agars after incubation for 14 days and 21 days, indicating the presence of slow-growing or potentially non-culturable bacteria in compost samples. Evidently, in dairy compost, some CE strains (n=12) were isolated only in the agar plates with Rpf, which was in line with the study reported by Su et al. (2018). According to Su et al. (2018), unique bacterial species belonged to genera *Bacillus*, *Arthrobacter*, *Nocardiopsis*, and *Mycobacterium* were isolated from agro-industrial compost only after Rpf addition because Rpf promoted the isolation of slow-growing bacteria from compost materials by significantly improving the cellulose-producing capability of bacterial community as well as facilitating the cell division. As a secretory protein of *M. luteus*, the mechanisms of Rpf involved in the resuscitation of VBNC bacterial cells is presumably initiated by lysis of cell wall, followed by releasing the peptidoglycan that can work as signaling molecules for the resuscitation of VBNC cells (Ramamurthy et al., 2014). Through the optimized culturing methods involving the use of Rpf with an extended incubation time, our results clearly demonstrated that animal-wastes-based compost is a promising source for isolating CE strains against *L. monocytogenes*.

In general, antagonistic activities of CE strains against pathogens can be quickly confirmed by spot-on-lawn for the isolates, disc diffusion, or agar-well diffusion methods for cell-free supernatant fluids (Ammor et al., 2006a). In this study, only 5 of 17 CE isolates showed the inhibition to selected *L. monocytogenes* when cell-free supernatants were tested. This discrepancy between isolates and supernatant testing results can be explained by the following reasons: 1) the antimicrobial compounds produced by CE strains may not

be accumulated to an adequate amount to induce the inhibition (Baindara et al., 2016); 2) the inhibition can only be effective when the cell is directly in contact with the pathogenic cell on agar surface but fail to release into the cell-free supernatant (Ammor et al., 2006ab). In this regard, the spot-on-lawn is a preferred method for quickly confirming the inhibitory activities caused by CE strains.

One of the selective criteria for CE cultures is the mobility, as the motility can contribute to the dispersal and afford the competitive advantage for bacteria when microbial members need to compete for limiting nutrient sources or attachment sites on the favorable surface (Hibbing et al., 2010). Microorganisms can compete passively by easy access to an attached space, and this "passive competition" can be achieved by the microbial motility (Bauer et al. 2018). For example, An et al. (2006) revealed that quorum-sensing-regulated functions and surface motility are important factors to enhance the microbial competition of *Pseudomonas aeruginosa* against *Agrobacterium tumefaciens* in biofilm cocultures. As such, it was not surprising that most motile CE isolates (all CE isolated from poultry compost) were classified as competitive biofilm former.

As the ability of *L. monocytogenes* to form biofilms can allow it to establish and persist for a long time in various environments, for a reasonable assumption, the CE strains with stronger biofilm-forming abilities might have advantage to prevent further biofilm formation by *L. monocytogenes*. On one hand, CE cultures may be directly applied to disrupt the pathogenic adhesion. In support to this assumption, Gueimonde et al. (2006) reported that the adhesion of *L. monocytogenes* on the mucosal surface was reduced by 19.1 to 25.9% by selected *Lactobacillus* strain. On the other hand, biofilms formed by CE

strains that already presented on a surface can potentially reduce the further colonization of *L. monocytogenes*. For example, populations of *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, and *Salmonella*, decreased by 0.4 – 2 log CFU/coupon after inoculation on the stainless-steel coupon contained the biofilm pre-formed by CE strains (Kim et al., 2013, Son et al., 2016, Turhan et al., 2017, Kim et al., 2018). Son et al. (2016) reported that the biofilm formed by CE cultures including *Streptomyces spororaveus, Bacillus safensis,* and *Pseudomonas azotoforman* on stainless steel with 7.9 to 8.5 log CFU/coupon of CE cultures reduced 1.8-1.9 log CFU/coupon of *S. aureus* depending on the CE strain used. Likewise, biofilms formed by *Lactobacillus* exhibited anti-*L. monocytogenes* activities, and caused a reduction of 0.7 to 2.0 log CFU/ml for planktonic *L. monocytogenes* and 0.4-1.7 log CFU/ml for *L. monocytogenes* biofilm. Most importantly, a broad spectrum of anti-*L. monocytogenes* activities from candidate CE strains isolated in this study was observed, suggesting these CE strains, after further characterization, can be a powerful tool for controlling *L. monocytogenes* in the preharvest environment.

Competitive exclusion microorganisms including most species identified in this study have been reported as possessing the inhibitory activities against foodborne pathogens. Some species belonging to *Bacillus* spp. can reduce the pathogen levels through the accumulation of several bacteriocins. As reported by Sabaté et al. (2013), surfactins produced by *Bacillus* subtilis C4, M1 and G2III were effective in the inhibition of *L. monocytogenes* by the well-diffusion method. Avci et al. (2016) reported that cell-free supernatants from *Bacillus* had a broad spectrum of antibacterial activity against *E. coli* O157: H7, *L. monocytogenes*, *Salmonella*, and *P. aeruginosa*. Besides, one species from

Bacillus spp., i.e. *Bacillus psychrodurans*, can inhibit the growth of *B. cereus, E. coli* O157: H7, *L. monocytogenes*, *Salmonella*, and *S. aureus* on agar plates, as determined by the spot-on-lawn method with diameter ranging from 9.58 ± 0.66 to 21.47 ± 0.27 mm for the inhibition zones (Das et al. 2017). In another study, Watabe et al. (2003) found that the growth of *Listeria ivanovii* was inhibited by *Paenibacillus lentimorbus* isolated from phase II mushroom compost, however, no quantity data was reported in that study. Additionally, *Planococcus* spp., which produces rhamnolipid biosurfactant, exhibited bactericidal activity against *L. monocytogenes* (Magalhães et al., 2013, Gaur et al., 2020). Although, the production of bacteriocin from *Brevibacillus* spp. and the probiotic properties of *Kocuria* spp. have been well-described in published studies (Baindara et al., 2016, Sharifuzzaman et al., 2018), to the best of our knowledge, this is the first time to report the inhibition of *L. monocytogenes* by both *Brevibacillus* spp. and *Kocuria* spp. In addition to have strong inhibitory activities against foodborne pathogens, CE strains identified in this study have been previously reported in numerous studies for controlling plant pathogens, including *Fusarium wilt*, *Aeromonas*, phytopathogenic fungi, and *Botrytis cinereal* in tomato or potato (Lee et al., 2006, Slimene et al., 2015, Lapidot et al., 2015, Li et al., 2019). Taken together, our results indicated that there was a prospective to use these CE strains as biological control agents for both plant and foodborne pathogens, like *L. monocytogenes* in the agricultural field. But it is noteworthy that the interactions between *L. monocytogenes* and CE microorganisms can be affected by their residential environments due to the variation in nutrient availabilities in these environments (Buchanan et al., 1999).

In order to simulate the nutrient conditions in the compost environment, CE and the tested *L. monocytogenes* strains were inoculated into the water extract of composts with different ratios. The efficacy of CE strains' antagonistic activity against *L. monocytogenes* increased with increasing nutrients at mesophilic temperature. As the CE strains were isolated and adapted from compost, they have higher growth rates than *L. monocytogenes* in compost extract (Figure 5.7), then out-compete the pathogen. Besides, the survival of *L. monocytogenes* inoculated in dairy or poultry wastes-based compost was significantly reduced in the presence of CE strains, implying the CE strains can effectively control *L. monocytogenes* during the storage of compost. The more reduction of *L. monocytogenes* in compost samples when co-culturing with CE strains may be due to the competition for the nutrients in compost and the release of antimicrobial compounds by CE strains. This conclusion can be supported by results from Chapter 4 of sequencing analysis that the microbial interactions were potentially due to the competition for nutrients within the compost environment, and the increased gene abundance in bacteriocins upon the introduction of *L. monocytogenes*.

Based on the findings from the co-culture study, there are several ways to apply the CE strains to the composts in order to provide another hurdle for reducing the level of *L. monocytogenes*. Firstly, the thermophilic CE strains may be added during the active composting process to work as probiotic-bioinoculant. Secondly, CE strains may be added during the curing phase, in order to prevent pathogen regrowth. Finally, CE inoculum can be added directly into the finished compost product just before land application, in order

to avoid the cross-contamination of *L. monocytogenes* and to enhance the biological control values to the final compost products.

LIMITATION

In the current study, the modified culture methods with extended incubation time were attempted to isolate compost-borne CE strains from different samples. However, some special incubation conditions, such as anaerobic or facultative anaerobic conditions were not included in this study. Therefore, one can assume that more CE strains may be isolated if these special conditions were used. Nevertheless, the isolation and screening methods used in this study can be more rapid and easily applied for isolating slow-grower CE strains from complex compost samples.

CONCLUSION

Our research demonstrated that the modified double/triple-agar-layer procedure coupled with spot-on-lawn testing can be a quick and efficient method for screening CE candidates from different compost samples, and that the addition of Rpf promotes the growth of slow-grower and potential viable but non-culturable species. The inhibition of *L. monocytogenes* by CE strains in both compost extracts and compost samples were observed at all incubation conditions and effected by compost types, nutrient levels, and incubation temperature. Findings from this chapter suggested the that animal waste-based compost is a promising and reliable source for isolating of CE microorganisms and adding specific CE microorganisms into composts is a practical approach to control *L.*

monocytogenes. For real-world applications, a cocktail of CE microorganisms might be applied to the compost during thermophilic or curing phases or a few days prior to agricultural land application of the finished compost. Furthermore, we believe that those identified CE microorganisms can be used in biological control of *L. monocytogenes* in various ecosystems.

ACKNOWLEDGMENT

We thank our industry collaborators for providing the compost samples used in this study. This research was financially supported by the Center for Produce Safety, University of California at Davis, and Specialty Crop Block Grant Program (SCBGP) of California Department of Food and Agriculture (CDFA–SCBGP) through a grant 1#8-0001-075-SC.

REFERENCES

1. Ammor S, Tauveron G, Dufour E, Chevallier I. 2006a. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1—Screening and characterization of the antibacterial compounds. Food Control 17:454-461.
2. Ammor S, Tauveron G, Dufour E, Chevallier I. 2006b. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 2—Behaviour of pathogenic and spoilage bacteria in dual species biofilms including a bacteriocin-like-producing lactic acid bacteria. Food Control 17: 462-68.

3. An D, Danhorn T, Fuqua C, Parsek MR. 2006. Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. Proc Natl Acad Sci U S A 103:3828-3833.
4. Avci A, Üzmez S, Alkan FB, Bagana İ, Nurçeli E, Çiftçi E. 2016. Antimicrobial activity spectrums of some *Bacillus* strains from various sources. GIDA/The Journal of food 41:5.
5. Baindara P, Singh N, Ranjan M, Nallabelli N, Chaudhry V, Pathania GL, Sharma N, Kumar A, Patil PB, Korpole S. 2016. Laterosporulin10: a novel defensin like class IId bacteriocin from *Brevibacillus* sp. strain SKDU10 with inhibitory activity against microbial pathogens. Microbiology 162:1286-1299.
6. Bauer MA, Kainz K, Carmona-Gutierrez D, Madeo F. 2018. Microbial wars: Competition in ecological niches and within the microbiome. Microb Cell 5:215-219.
7. Buchanan R, Bagi L. 1999. Microbial competition: effect of *Pseudomonas fluorescens* on the growth of *Listeria monocytogenes*. Food Microbiol 16:523-529.
8. California leafy greens marketing agreement 2010. https://lgma-assets.sfo2.digitaloceanspaces.com/downloads/Appendix_E.pdf Accessed on Mar. 2020
9. Chen H, Fu L, Luo L, Lu J, White WL, Hu Z. 2012. Induction and resuscitation of the viable but nonculturable state in a cyanobacteria-lysing bacterium isolated from cyanobacterial bloom. Microb Ecol 63:64-73.

10. Das G, Park S, Baek K. 2017. Diversity of endophytic bacteria in a fern species *Dryopteris uniformis* (Makino) Makino and evaluation of their antibacterial potential against five foodborne pathogenic bacteria. Foodborne Pathog. Dis. 14:260-268.
11. Gaur VK, Tripathi V, Gupta P, Dhiman N, Regar RK, Gautam K, Srivastava JK, Patnaik S, Patel DK, Manickam N. 2020. Rhamnolipids from *Planococcus* spp. and their mechanism of action against pathogenic bacteria. Bioresour Technol 123206.
12. Gensler CA, Brown SR, Aljasir SF, D'amico DJ. 2020. Compatibility of commercially produced protective cultures with common cheesemaking cultures and their antagonistic effect on foodborne pathogens. J Food Prot 83:1010-1019.
13. Gueimonde M, Jalonen L, He F, Hiramatsu M, Salminen S. 2006. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. Food Res Int 39:467-471.
14. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology 8:15-25.
15. Jiang X, Shepherd M. Fan X, Niemira B.A, Doona C.J, Feeherry F.E, Gravani R.B (Eds.). 2009. Microbial Safety of Fresh Produce, Blackwell Publishing p. 143
16. Jin Y, Gan G, Yu X, Wu D, Zhang L, Yang N, Hu J, Liu Z, Zhang L, Hong H. 2017. Isolation of viable but non-culturable bacteria from printing and dyeing wastewater bioreactor based on resuscitation promoting factor. Curr Microbiol 74:787-797.
17. Kato S, Yamagishi A, Daimon S, Kawasaki K, Tamaki H, Kitagawa W, Abe A, Tanaka M, Sone T, Asano K, Kamagata Y. 2018. Isolation of previously uncultured

slow-growing bacteria by using a simple modification in the preparation of agar media. Appl Environ Microbiol 84:10.

18. Kim J, Jiang X. 2010. The growth potential of *Escherichia coli* O157: H7, *Salmonella* spp. and *Listeria monocytogenes* in dairy manure-based compost in a greenhouse setting under different seasons. J Appl Microbiol 109:2095-2104.
19. Kim M, Morrison M, Yu Z. Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. 2011. J. Microbiol. Methods. 84(1):81-7.
20. Kim S, Bang J, Kim H, Beuchat LR, Ryu J. 2013. Inactivation of *Escherichia coli* O157: H7 on stainless steel upon exposure to *Paenibacillus polymyxa* biofilms. Int J Food Microbiol 167:328-336.
21. Kim Y, Kim H, Beuchat LR, Ryu J. 2018. Development of non-pathogenic bacterial biofilms on the surface of stainless steel which are inhibitory to *Salmonella enterica*. Food Microbiol 69:136-142.
22. Lapidot D, Dror R, Vered E, Mishli O, Levy D, Helman Y. 2015. Disease protection and growth promotion of potatoes (*S olanum tuberosum L*.) by *Paenibacillus dendritiformis*. Plant Pathol 64:545-551.
23. Lee JP, Lee S, Kim CS, Son JH, Song JH, Lee KY, Kim HJ, Jung SJ, Moon BJ. 2006. Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. Biol. Control 37:329-337.

24. Li B, Wang B, Pan P, Li P, Qi Z, Zhang Q, Shi C, Hao W, Zhou B, Lin R. 2019. *Bacillus altitudinis* strain AMCC 101304: a novel potential biocontrol agent for potato common scab. Biocontrol Sci Technol 29:1009-1022.

25. Magalhães L, Nitschke M. 2013. Antimicrobial activity of rhamnolipids against *Listeria monocytogenes* and their synergistic interaction with nisin. Food Control 29:138-142.

26. Muñoz N, Sonar CR, Bhunia K, Tang J, Barbosa-Cánovas GV, Sablani SS. 2019. Use of protective culture to control the growth of *Listeria monocytogenes* and *Salmonella typhimurium* in ready-to-eat cook-chill products. Food Control 102:81-86.

27. Nurmi E, Nuotio L, Schneitz C. 1992. The competitive exclusion concept: development and future. Int J Food Microbiol 15:237-240.

28. Oliveira M, Usall J, Viñas I, Solsona C, Abadias M. 2011. Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves. Food Microbiol 28:590-596.

29. O'Toole GA. 2011. Microtiter dish biofilm formation assay. JoVE (Journal of Visualized Experiments) e2437.

30. Pinto D, Almeida V, Almeida Santos M, Chambel L. 2011. Resuscitation of *Escherichia coli* VBNC cells depends on a variety of environmental or chemical stimuli. J Appl Microbiol 110:1601-1611.

31. Powell J, Witthuhn R, Todorov S, Dicks L. 2007. Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. Int Dairy J 17:190-198.

32. Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S. 2014. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Front. Public Health. 2:103.

33. Sabaté DC, Audisio MC. 2013. Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. subtilis strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. Microbiol Res 168:125-129.

34. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15.

35. Sharifuzzaman S, Rahman H, Austin DA, Austin B. 2018. Properties of probiotics *Kocuria* SM1 and *Rhodococcus* SM2 isolated from fish guts. Probiotics Antimicrob. Proteins 10:534-542.

36. Shields P, Cathcart L. 2011. Motility test medium protocol. ASM https://www.asmscience.org/content/education/protocol/protocol.3658?crawler=redirect&mimetype=application/pdf. Accessed on Aug. 2020.

37. Shukla SK, Rao TS. 2017. An improved crystal violet assay for biofilm quantification in 96-well microtitre plate. Biorxiv 100214.

38. Slimene IB, Tabbene O, Gharbi D, Mnasri B, Schmitter JM, Urdaci M, Limam F. 2015. Isolation of a chitinolytic *Bacillus licheniformis* S213 strain exerting a biological control against *Phoma medicaginis* infection. Appl Biochem Biotechnol 175:3494-3506.

39. Son H, Park S, Beuchat LR, Kim H, Ryu J. 2016. Inhibition of *Staphylococcus aureus* by antimicrobial biofilms formed by competitive exclusion microorganisms on stainless steel. Int J Food Microbiol 238:165-171.
40. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 40:175-179.
41. Su X, Zhang S, Mei R, Zhang Y, Hashmi MZ, Liu J, Lin H, Ding L, Sun F. 2018. Resuscitation of viable but non-culturable bacteria to enhance the cellulose-degrading capability of bacterial community in composting. Microbial biotechnology 11:527-536.
42. Turhan EU, Erginkaya Z, Uney MH, Ozer EA. 2017. Inactivation effect of probiotic biofilms on growth of *Listeria monocytogenes*. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 23:.
43. Van Houdt R, Michiels C. 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol 109:1117-1131.
44. Watabe M, Rao JR, Murphy AR, Moore JE. 2003. Inhibition of *Listeria ivanovii* by *Paenibacillus lentimorbus* isolated from phase II mushroom compost. World J. Microbiol. Biotechnol. 19:875.
45. Weis J, Seeliger HP. 1975. Incidence of *Listeria monocytogenes* in nature. Appl Microbiol 30:29-32.

46. Zhao T, Podtburg TC, Zhao P, Schmidt BE, Baker DA, Cords B, Doyle MP. 2006. Control of *Listeria* spp. by competitive-exclusion bacteria in floor drains of a poultry processing plant. Appl Environ Microbiol 72:3314-3320.

Table 5.1 *L. monocytogenes* and surrogate strains used in this study

Label	FSL-ID	Serotype (lineage, clonal complex)	Previous strain ID (provided by)	Isolate source [b]
L1	*L.monocytogenes* R2-0503	1/2b (I, CC3)	(ILSI collection)	Human, 1994
L2	*L.monocytogenes* J1-0107	4d (I, CC1)	N. A	Coleslaw
L3	*L.monocytogenes* J1-0101	1/2a (II, CC11)	(ILSI collection)	Hot dog
L4	*L.monocytogenes* R9-0506	1/2a (II, CC29)	L25265 (CDC)	Cantaloupe, 2011
L5	*L.monocytogenes* R9-5506	4b (I, ST382)	PNUSAL001751 (CDC)	Packaged salad, 2016
L6	*L.monocytogenes* R9-5507	4b (I, CC554)	PNUSAL000954 (CDC)	Sprouts, 2014
L7	*L.monocytogenes* J1-0031	4a (II, CC396)	(ILSI collection)	Human
L8	*L.monocytogenes* J1-0158	4b (IV, ST382)	(ILSI collection)	Goat
L9	*L.monocytogenes* S10-2161	1/2a (II, ST364)	N. A	Soil spinach field
L10	*L. innocua* C2-0008	N. A[a]	N. A	Fish processing plant, 2000

[a] Not available.

[b] Indicates outbreak-associated sources.

Table 5.2 New bacterial colonies counted on PYG agar plates from the highest countable dilution on days 14 and 21

	New colonies (CFU/plate)	
Collection farm	Day 14 (% of increased) [a]	Day 21(% of increased)
Poultry farm #1	17 (9.5%)	10 (5.6%)
Poultry farm #2	42 (22.1%)	31 (30.0%)
Poultry farm #3	25 (156.3%)	7 (43.8%)
Dairy farm #1	9 (8.4%)	4 (3.7%)
Dairy farm #2	16 (11.0%)	21 (14.5%)
Dairy farm #3	43 (31.6%)	57 (41.9%)

[a] % of increase as compared to the colonies appeared on Day 7 of highest countable dilution plate.

Table 5.3 Characteristics of candidate CE strains isolated from animal waste-based composts

Isolates #	**Compost**	**Agar**	***L. monocytogenes* or surrogate strains**													**Motile**	**Gram-staining**	**Growth at 55°C**
			101M	LCDC 81-861	Scott A	1	2	3	4	5	6	7	8	9	*L.innocua*			
P1	Poultry farm 3	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	Yes	Variable/ rod	No
P2	Poultry farm 3	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	Yes	Variable/ rod	No
P3	Poultry farm 3	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	Yes	G+, rod	Yes
P4	Poultry farm 3	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	Yes	G+, rod	Yes
P5	Poultry farm 3	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	Yes	G+, rod	No
P6	Poultry farm 3	TSA	+	-	+	+	+	+	+	+	+	+	+	+	+	Yes	G+, rod	Yes (Weak growth [b])
P7	Poultry farm 1	TSA	-	+	-	+	+	+	+	+	+	+	+	+	+	Yes	G+, rod	No
P8	Poultry farm 1	TSA	+	-	-	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes
P9	Poultry farm 1	TSA	+	-	-	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes
P10	Poultry farm 1	TSA	+	-	-	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes

P11	Poultry farm 1	TSA	+	-	-	+	+/- [a]	+ /-	+/ -	-	+/ -	-	-	+/ -	-	No	G+, rod	Yes
P12	Poultry farm 1	TSA	+	-	-	+	+	+	+	-	+	-	-	+	-	No	G+, rod	Yes
P13	Poultry farm 1	TSA	+	-	-	+	+	+	+	-	+	-	-	+	-	Yes	G+, rod	Yes
P14	Poultry farm 1	TSA	-	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes
P15	Poultry farm 2	TSA	+	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes
D1	Dairy farm 3	TSA	+	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, coccus	Yes (Weak growth)
D2	Dairy farm 1	TSA	+	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	No
D3	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, coccus	No
D4	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, rod	No
D5	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	-	+	+	+	+	+	Yes	G+, rod	No
D6	Dairy farm 3	MPN + Rpf	+	+	+	-	-	-	-	-	-	-	-	+	-	Yes	G+, rod	No
D7	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, rod	No

D8	Dairy farm 1	TSA	-	+	+	-	+/-	-	+	+/-	+	-	-	+/-	+	No	G+, coccus	No
D9	Dairy farm 1	MPN + Rpf	-	+	+	-	+/-	-	+	+/-	+	-	-	+/-	+	No	G+, coccus	No
D10	Dairy farm 1	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, rod	No
D11	Dairy farm 1	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, coccus	No
D12	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D13	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D14	Dairy farm 1	TSA	-	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	No
D15	Dairy farm 1	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	-	Yes	G+, rod	Yes (Weak growth)
D16	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D17	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, coccus	No
D18	Dairy farm 3	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, coccus	No

D19	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D20	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D21	Dairy farm 1	TSA	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, rod	No
D22	Dairy farm 1	MPN + Rpf	+	+	+	+	+	+	+	+	+	+	+	+	No	G+, rod	No
D23	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D24	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	No	G+, coccus	No
D25	Dairy farm 3	MPN + Rpf	-	+	+	-	-	-	-	-	-	-	-	-	No	G+, coccus	No

[a] +/- indicated that the zone-of- inhibition was not very clear.

[b] weak growth was defined as very little biomass was shown on the plates.

Figure 5.1 Flow-chart for testing candidate CE strains co-culturing with *L. monocytogenes* in sterile compost extracts and solid compost samples.

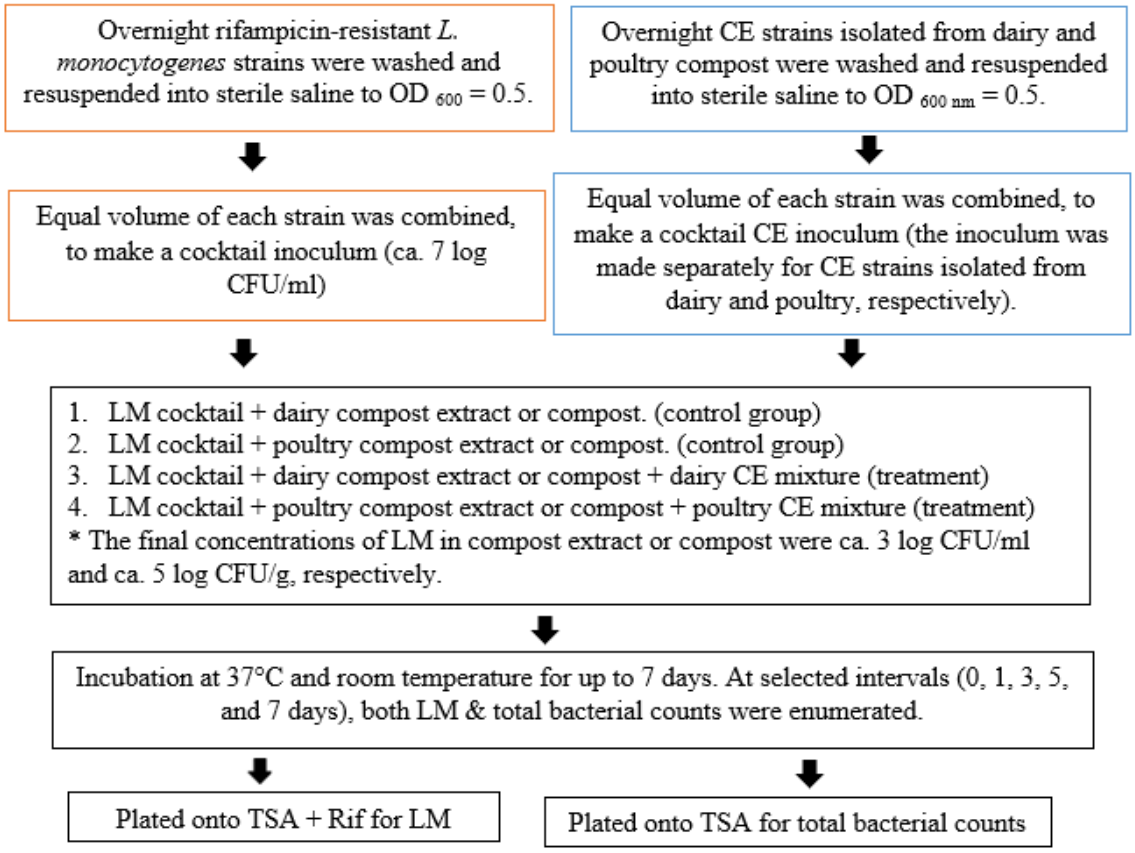


Figure 5.2 Selection of competitive exclusion microorganisms from compost samples against *L. monocytogenes*. The isolates showed no inhibition zone on *Listeria* lawn (A), and with various sizes of inhibition zones (B). And, the selected plate showed results from well-diffusion results (C); well-1 showed clear zone of inhibition, whereas wells 2-5 showed very light zones of inhibition. The negative control with sterile 0.85% saline didn't have a zone of inhibition.

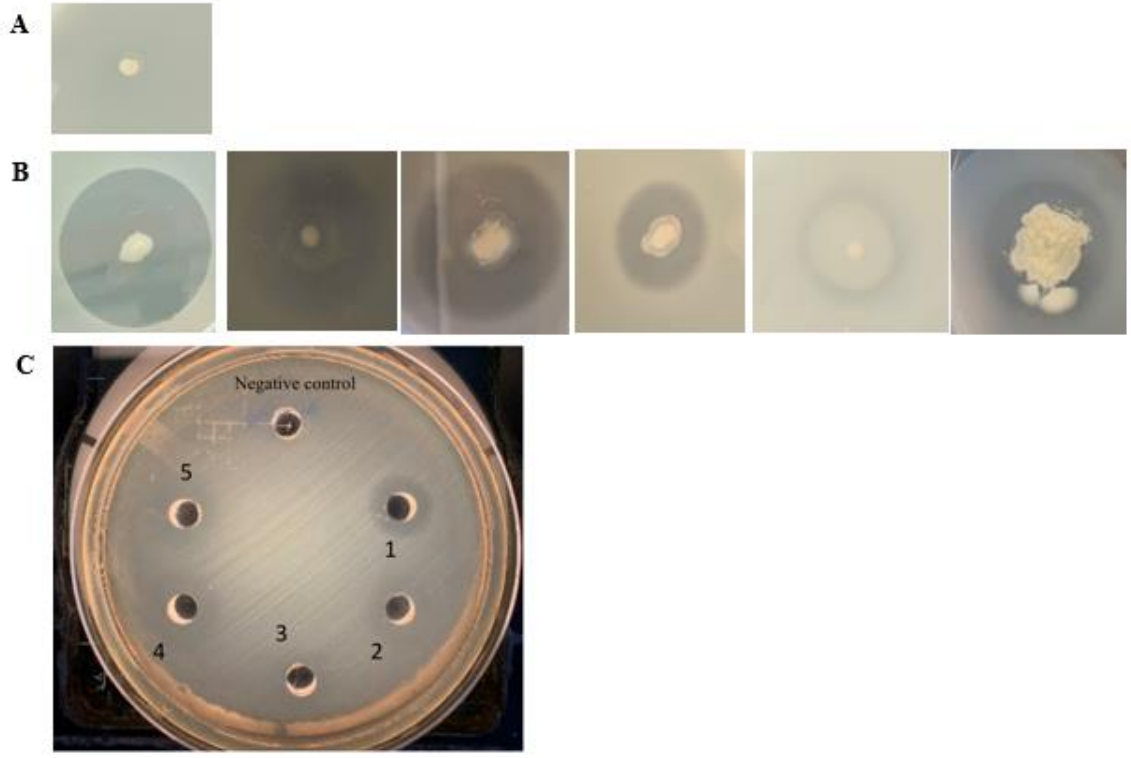


Figure 5.3 Biofilm-forming ability of *L. monocytogenes* strains (L1 – L10) (A) and *L. monocytogenes* strain control L9 and CE strains (D1-P15) (B) as determined by crystal violet assay. Strains labeled with square formed significantly ($P < 0.05$) more biofilm than *L. monocytogenes* control strain L9 did.

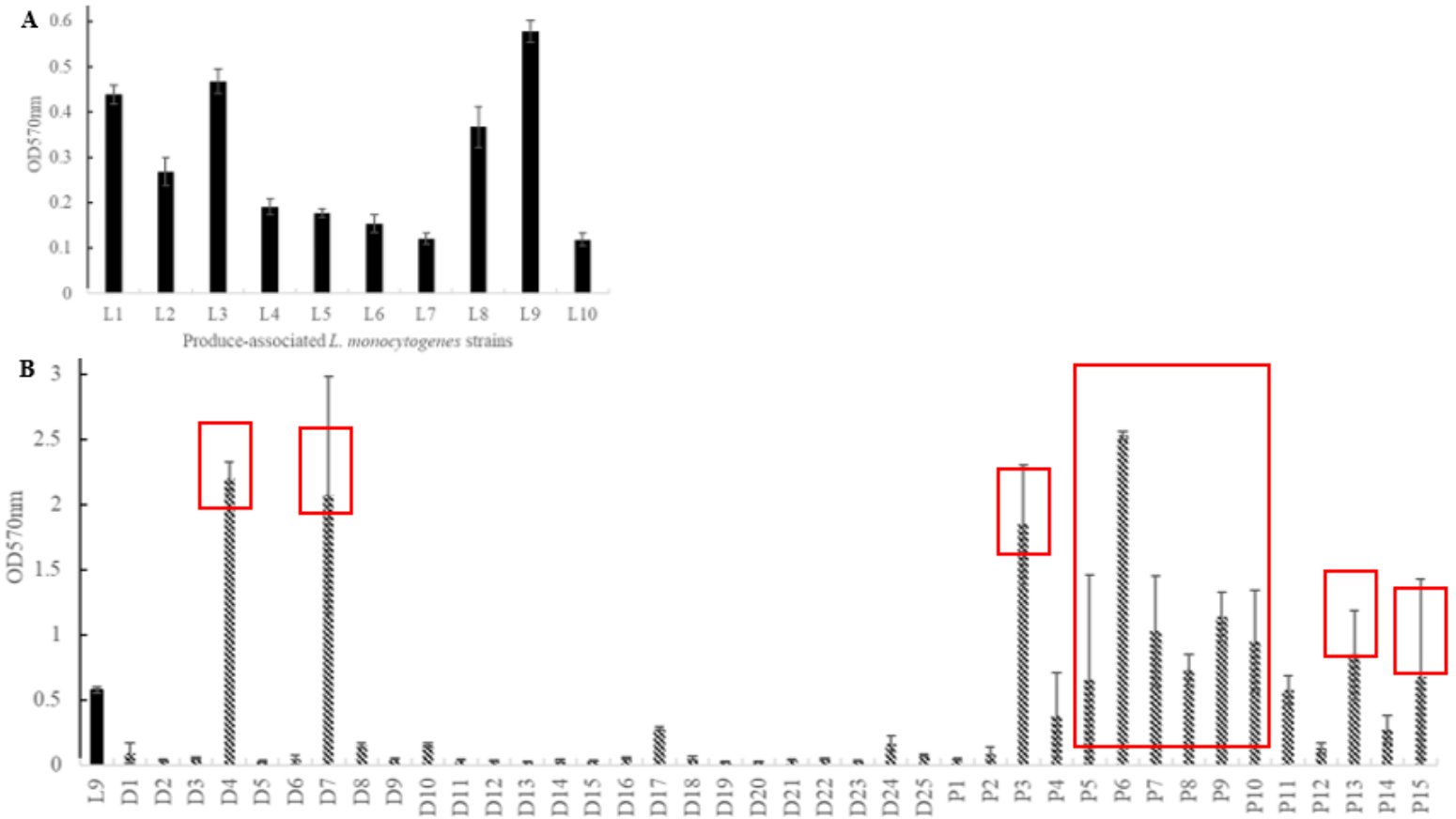


Figure 5.4 Inhibition of 3 *L. monocytogenes* strains at the presence of CE in 1:5 and 1:10 dairy compost extracts at 35°C and room temperature (RT), respectively. Data are expressed as average log CFU/ml from two separate trials.

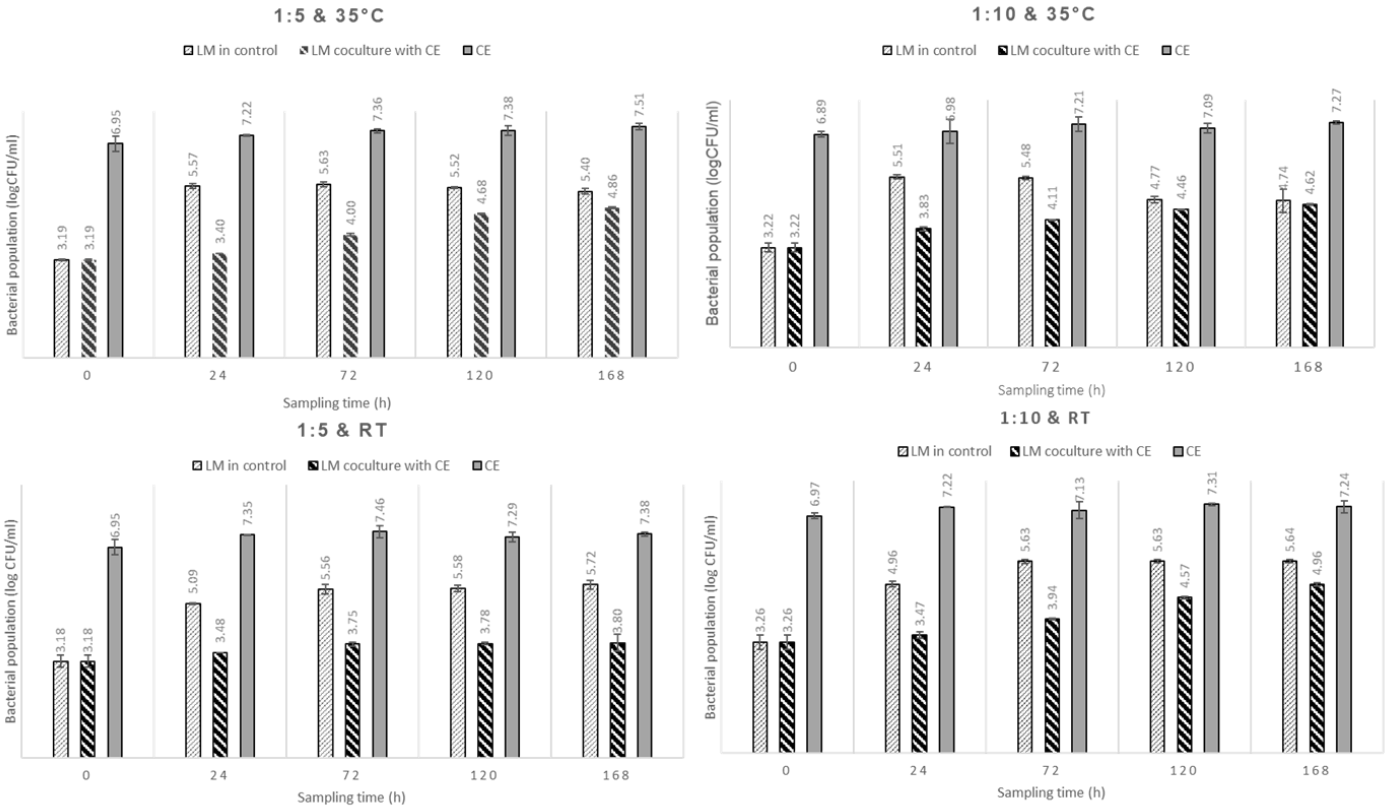


Figure 5.5 Inhibition of 3 *L. monocytogenes* strains at the presence of CE in 1:5 and 1:10 poultry compost extracts at 35°C and room temperature (RT), respectively. Data are expressed as average log CFU/ml from two separate trials.

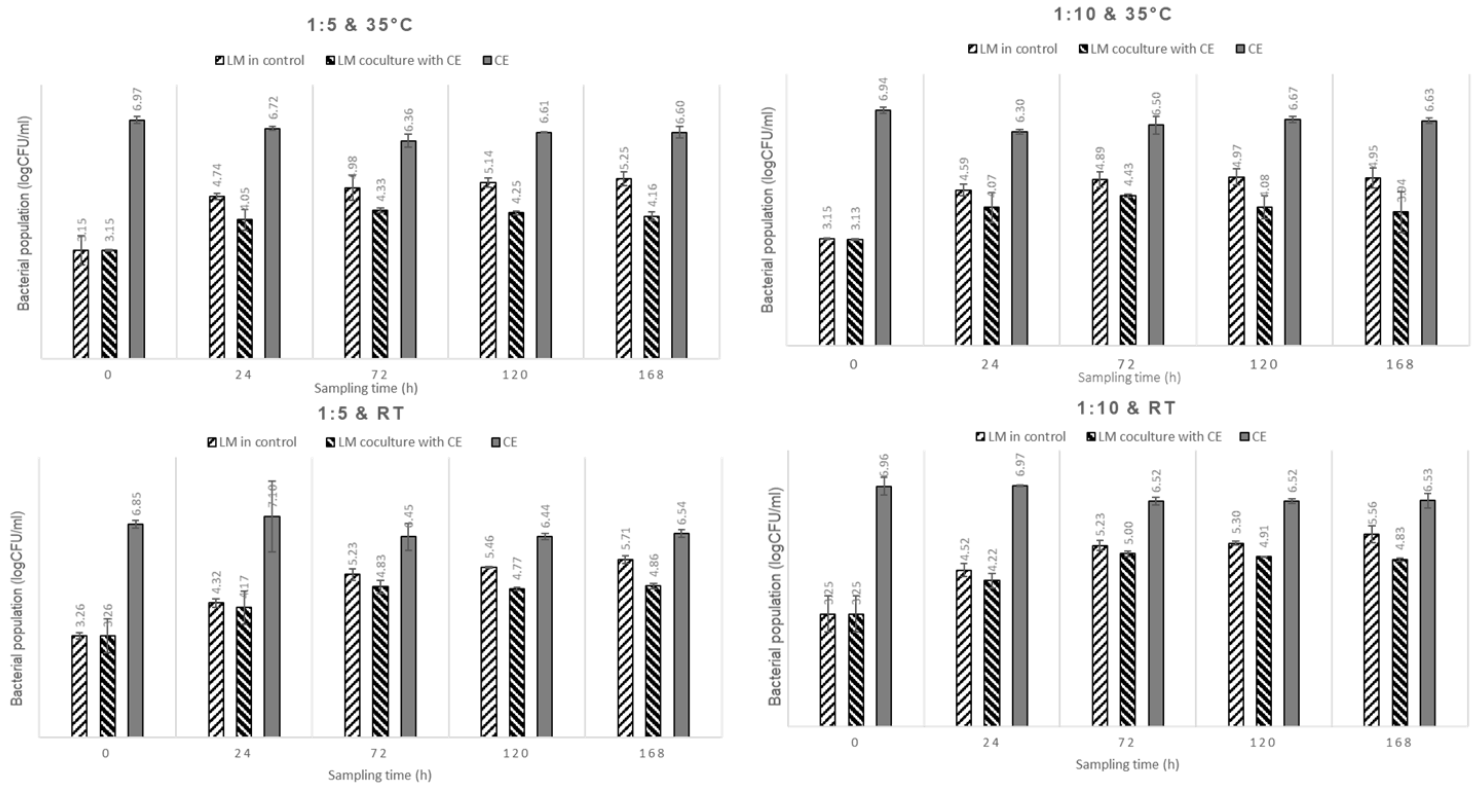


Figure 5.6 Inhibition of 3 *L. monocytogenes* strains at the presence of CE in dairy (A) and poultry compost (B) at room temperature, respectively. Data are expressed as average log CFU/g in dry weight from two separate trials.

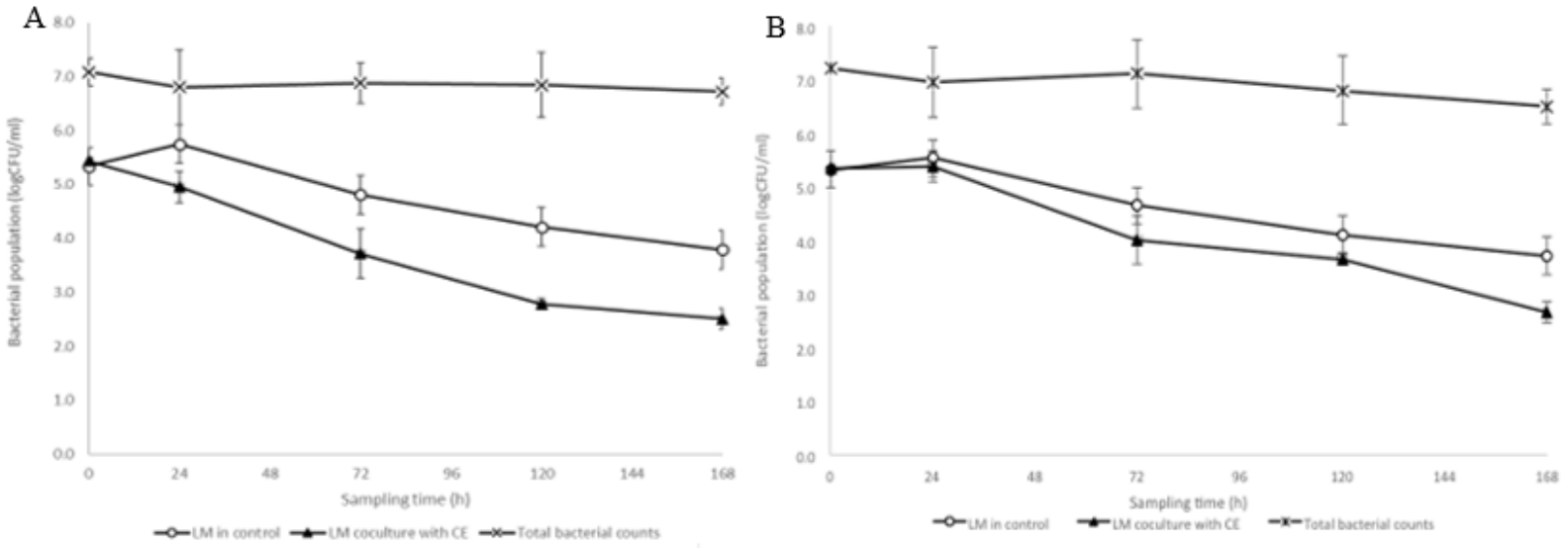
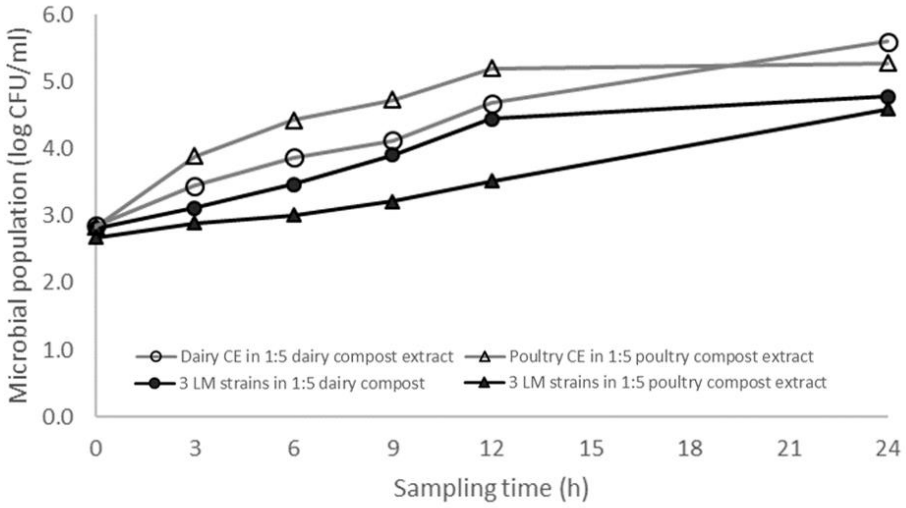


Figure 5.7 Growth rates of three-strain cocktail of *L. monocytogenes* strains, poultry or dairy CE in compost extracts.



CONCLUSIONS

Animal waste or animal waste-based compost are commonly used as organic fertilizer to grow fresh-produce. To reduce harmful microorganisms, animal wastes can be treated by composting or other validated scientific methods. But the insufficient treatment may introduce pathogens, such as *Salmonella* spp. or *L. monocytogenes* to produce production. Physical heat treatments are commonly used to kill *Salmonella* in poultry litter with or without composting process, but the validation study is still needed for the litter processing industry to ensure microbial safety of their products. Further, due to the ubiquitous nature of *L. monocytogenes*, it is essential to understand the ecology of this pathogen where it inhabits and then develops strategies to reduce *Listeria* contamination. We thus hypothesized that the compost-adapted competitive exclusion (CE) microorganisms against *L. monocytogenes* exist in animal waste-based compost. In combination with the culturing method, the use of next-generation sequencing approaches is expected to speed up the discovery of those compost-borne CE microorganisms for controlling *L. monocytogenes* in pre-harvest environments.

Our lab-based study revealed that that 1.2- to 2.7-log or more reductions of desiccation-adapted *E. faecium* NRRL B-2354 were equivalent to > 5-log reductions of desiccation-adapted *Salmonella* Senftenberg 775/W in poultry litter compost, depending on treatment conditions and compost types. The thermal processes of two poultry litter processing plants have been successfully validated using *E. faecium* NRRL B-2354 and presumptive indigenous enterococci. The validation results showed that *Salmonella* levels

could be reduced by at least 5-log according to the reductions of surrogate and indicator microorganisms.

Based on the NGS results, the microbial compositions ad functions in compost were affected by the compost-related factors, but not by the introduction of *L. monocytogenes*. The interactions between *L. monocytogenes* and the native microbial members were generally limited and did not affect the dominant members of the microbial community in the compost ecosystem, but some discriminatory species were identified. Additionally, we have demonstrated that animal waste-based compost is a promising and reliable source for isolation of CE microorganisms. A cocktail of CE microorganisms might be applied to the compost during composting or a few days prior to agricultural land application of the finished compost.

In summary, we have validated current processes for physically heat-treated poultry litter in industry settings, and provided tools (surrogate and/or indicator microorganism for *Salmonella*, and custom-designed sampler) for litter processors to modify their existing process parameters to reduce the *Salmonella* level in physically heat-treated poultry litter compost, which can be used by the produce industry to grow microbiologically safe products. Besides, findings from NGS research confirmed that commercial compost products contain a diversity of microbial species including CE species. The compost-adapted CE microorganisms can be used as biological tools to control *L. monocytogenes* contamination in produce growing and processing environments. Further studies related to the applications of CE strains on controlling foodborne pathogens on farm or processing environments are needed. The compositional and functional changes in microbial

communities of compost spiked with CE strains could also be investigated by metagenomic sequencing analysis in a field study.

APPENDICES
Appendix A

Search strings and preferred reporting items for systematic reviews and meta-analyses (PRISMA) for systematic literature search

Table A1 Search strings for factors influencing the survival of *Salmonella* or *L. monocytogenes* in animal wastes or animal waste-based compost

PATHOGENS		ANIMAL WASTES TYPE		ROLE
Salmonella OR		Animal waste OR		Persistence OR
Listeria monocytogenes	**AND**	Animal manure OR	**AND**	Survival OR
		Animal litter OR		Growth OR
		Animal waste-based compost		Fate OR

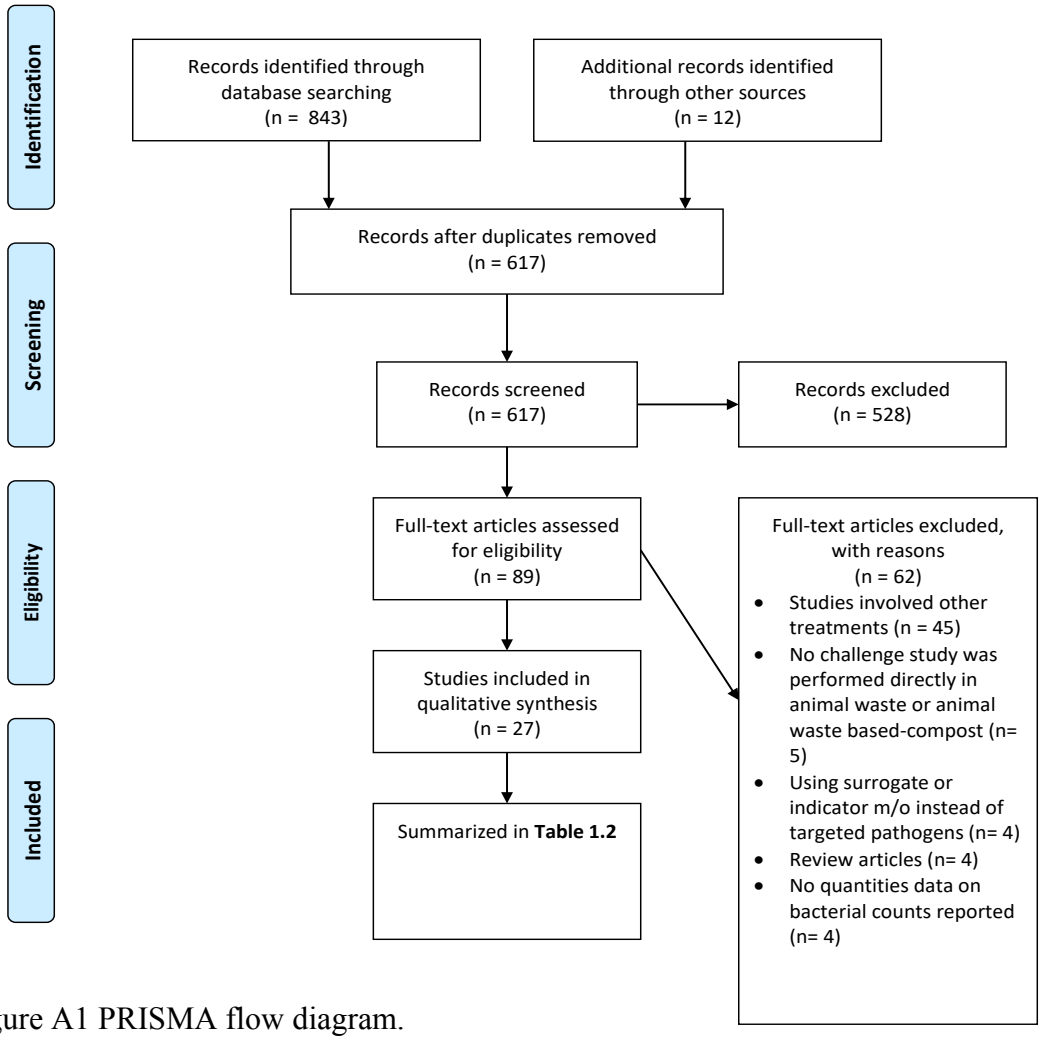


Figure A1 PRISMA flow diagram.

Table A2 Search strings for the efficacy of using surrogate microorganisms to validate thermal inactivation of *Salmonella* spp. in different study matrix

PATHOGENS		MICROORGANISMS		TREATMENT
Salmonella		Surrogate		Thermal inactivation OR
	AND		**AND**	Thermal treatment OR
				Heat inactivation OR
				Heat treatment

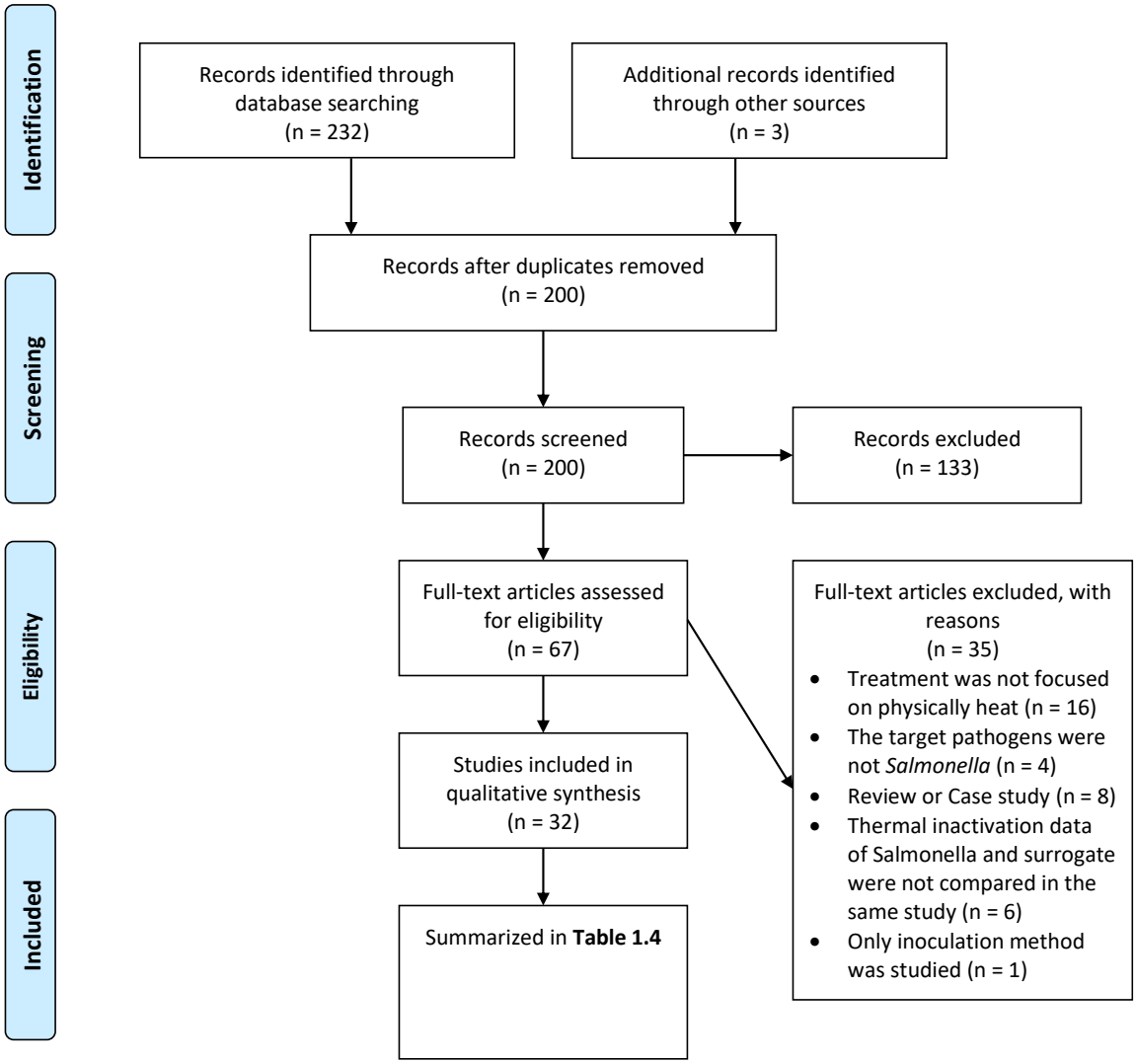


Figure A2 PRISMA flow diagram.

Table A3 Search strings for identifying competitive exclusion strategies used to control major foodborne pathogens from farm to food processing plants

PATHOGENS		STRATEGIES		APPLICATION FIELD
Salmonella OR	AND	Competitive exclusion OR	AND	Fresh produce OR
Listeria OR		Biological control OR		Crops OR
Pathogenic *E. coli*		Antagonistic		Plant OR
				Soil OR
				Rhizosphere OR
				Food processing OR
				Food facility OR
				Meat plant OR
				Biofilm

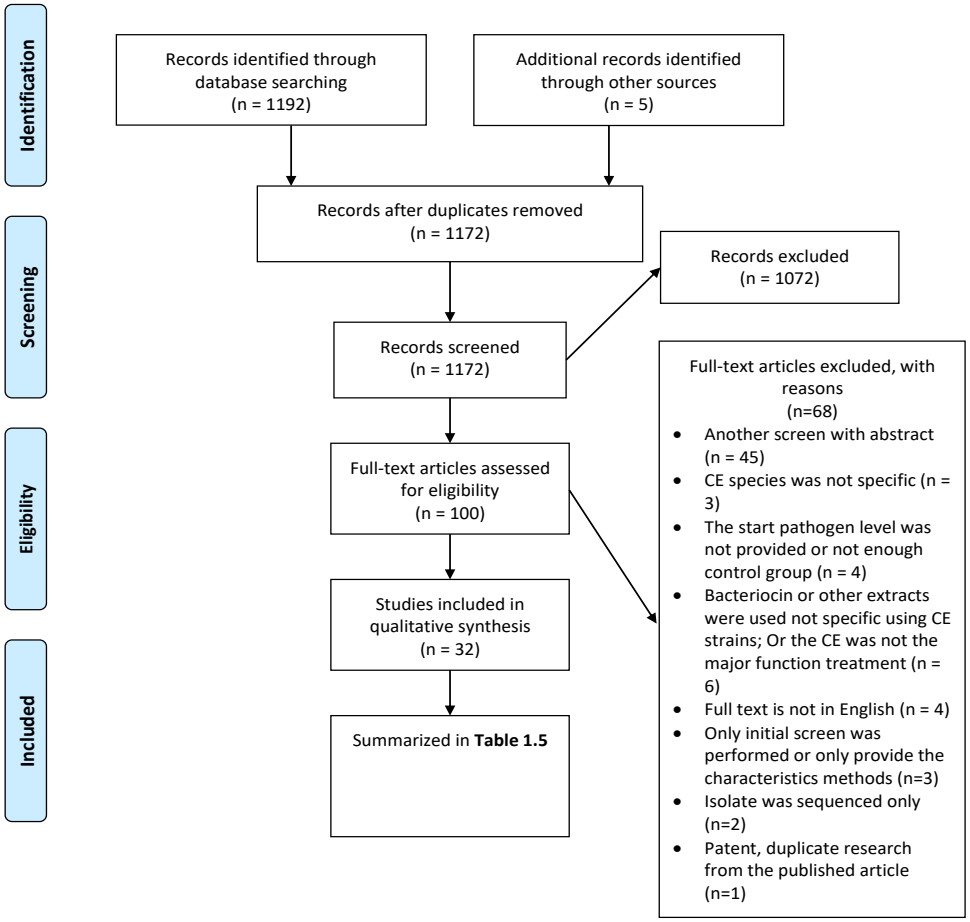


Figure A3 PRISMA flow diagram