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Project Title
Identifying competitive exclusion organisms against *Listeria monocytogenes* from biological soil amendments by metagenomic, metatranscriptomic, and culturing approaches

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Objectives

1. *Analyze microbial community structure of a variety of biological soil amendments using phylogenetic marker analysis based on the sequencing of 16S rRNA for bacteria and 18S rRNA for eukaryotes in the presence and absence of Listeria monocytogenes.*
2. *Analyze functional metatranscriptomics of L. monocytogenes interactions with indigenous compost microorganisms to identify competitive exclusion (CE) microorganisms for antagonistic activity against the pathogen.*
3. *Optimize culturing conditions to isolate or validate CE microorganisms against L. monocytogenes.*

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FINAL REPORT

Abstract

Listeria monocytogenes is a leading foodborne pathogen that may contaminate fresh produce in both farming and food processing environments, resulting in deadly outbreaks. To reduce *Listeria* contamination, it is essential to understand the ecology of this pathogen where it inhabits, and then develop strategies for pathogen control. Composts are a rich source of microorganisms with a diversity of microbial species, so compost-adapted competitive exclusion (CE) microorganisms against *L. monocytogenes* are expected to exist. In this project, we used targeted, metagenomic, and metatranscriptomic sequencing approaches (**Obj. I** & **II**) along with culturing methods (**Obj. III)** to achieve the research goals.

For Obj. I, 12 biological soil amendments (6 dairy and 6 poultry waste–based composts, at both active and finished stage) were obtained from six compost facilities. The thoroughly mixed compost samples were adjusted to moisture contents of 40 and 80%, and then inoculated with *L. monocytogenes* strain FSL R9-5506, with optimized inoculation conditions (ca. 7 log CFU/g) as determined by denaturing gradient gel electrophoresis (DGGE). At 0 and 72 h incubation, *L. monocytogenes* populations in the compost samples were enumerated, and the genomic DNA was extracted, purified, and then subjected to phylogenetic marker analysis based on the sequencing of 16S rRNA for bacteria and 18S rRNA for fungi. During 72 h incubation at room temperature, the inoculated *L. monocytogenes* population was either reduced or increased depending on the compost type and moisture levels. Through targeted sequencing analysis, the top dominant bacterial and fungal species were identified from poultry and dairy composts, and the indigenous bacterial communities across compost samples were analyzed. For instance, the discriminant microbial species at the genus level were more enriched in active dairy compost (18 genera) as compared with those in the finished dairy, or active and finished poultry composts. Fifteen genera used as biological control agents for preventing plant or soil pathogens were identified in the compost samples by 16S targeted sequencing. Furthermore, the microbial compositions of *L. monocytogenes*–inoculated and uninoculated compost samples were extensively analyzed by powerful multivariate statistical approaches. The microbial community of samples was separated by compost types and farms, and samples with *Listeria* inoculation did separate from those without, albeit within each compost type from the same farm. Although there was no direct evidence that the compost microbial composition was significantly changed by the introduction of *L. monocytogenes*, the interactions between the discriminant microbial members and *L. monocytogenes* were found to vary in different compost samples, suggesting that the microbial interactions were influenced by their habitable environments. Among the top 20 discriminatory bacterial genera, *Ureibacillus*, *Sphaerobacter*, and *Bacillus* were detected in all dairy compost samples, whereas *Bacillus* and *Paucisalibacillus* were detected in most poultry compost samples, indicating there was a higher chance to isolate competitive exclusion (CE) strains by culturing methods from these genera.

The active compost samples collected from dairy farm #1 were selected for the Obj. II study as more potential CE microorganisms were identified in this compost. Immediately after the composts from two collections were received, *L. monocytogenes* strain FSL R9-5506 was inoculated into compost samples with 80% moisture content, as described for Obj. I. After 0 and 72 h incubation periods, both DNA and RNA samples were extracted, purified, and submitted to Novogene Inc. for metagenomic and metatranscriptomic sequencing, respectively. Sequencing outputs were uploaded to MG-RAST for taxonomic and functional assignations of the sequences, followed by statistical analysis using STAMP software. As revealed by

metagenomic sequencing analysis, the five most abundant phyla accounting for at least 91% of all classified reads in the active dairy compost samples were in agreement with the results from Obj. I. The functional capacities of composts from two collections were statistically compared based on SEED subsystem level 1, and 8 of the 28 functional profiles were found significantly different ($P < 0.05$) between the two collections, suggesting the heterogeneous mixtures of compost samples. From the metatranscriptomic sequencing data, we also compared the gene expression changes associated with specific microbial functional pathways in compost with and without *L. monocytogenes* at 0 and 72 h incubation periods, and 75 of 197 pathways of active functional categories found at the SEED subsystem level 2 differed among different treatment groups. After 72 h, changes in the metabolic pathways and the increased abundance of the bacteriocins category in the compost samples containing *L. monocytogenes* indicate that the interactions between *L. monocytogenes* and compost microflora may include competition for compost nutrients and the presence of antimicrobials produced by CE bacteria. This observation confirmed that active compost collected from dairy farm #1 was a good candidate for isolating CE microorganisms in Obj. III.

Based on the results of Obj. I, compost samples with a high abundance of potential CE microorganisms were plated onto nutrient agar, proteose-yeast-glucose (PYG), and most probable number + resuscitation promoting factor agar plates (MPN + Rpf). Following the different incubation conditions, CE strains were screened and isolated from composts using double- or triple-agar-layer methods, and then identified to species level by Sanger sequencing (Obj. III). Through different culturing methods**,** a total of 40 bacterial isolates were confirmed with anti–*L. monocytogenes* activities. In agreement with Obj. I results, 50% of the isolated CE strains by culturing methods were identified as *Bacillus*. The 40 CE isolates were separated into two groups, i.e. poultry and dairy CE groups, and then tested for anti–*L. monocytogenes* activity in a compost extract model. Briefly, the filter-sterilized compost extracts inoculated with a cocktail of three *L. monocytogenes* strains (ca. 3 log CFU/ml) were mixed with or without CE (ca. 7 log CFU/ml), and incubated at 22°C (room temperature) and 35°C up to 168 h. At selected intervals, samples were enumerated for *L. monocytogenes* and CE populations, respectively. The growth of *L. monocytogenes* was reduced by co-culturing with CE strains in both dairy and poultry compost extracts under all incubation conditions, and the inhibition effect from CE strains increased in more concentrated compost extract (1:5) at 35°C. Findings from this project revealed the microbial compositions of indigenous microflora in a variety of compost samples, and identified CE microorganisms that potentially can be used as biological tools to control *L. monocytogenes* contamination in produce growing and processing environments.

Background

Listeria monocytogenes is widespread in the environment, and has been reported to survive in sewage sludge, plants and decaying vegetation, soil, animal hives and feces (Weis and Seeliger, 1975). The bacterium can adapt as a “resident” microorganism in many environments when living as a saprophyte as well as transitioning to the life within the host. Animal wastes are the major source of many human pathogens, such as *L. monocytogenes*, *Escherichia coli* O157:H7, *Salmonella enterica*, and so on, which can survive for extended periods of time or even multiply (Jiang and Shepherd, 2009). For example, Oliveira et al. (2011) reported that *Listeria* could be transferred from soil contaminated with organic compost to lettuce leaves, indicating that contaminated biological soil amendments can play an important role in contaminating fresh produce. One of the most effective and harmless methods available to control pathogens is competitive exclusion (CE). There is strong interest in applying CE

microorganisms as biological control tools for pathogen reduction in food, agricultural, and medical fields. The treatment is totally biological, as it does not leave any harmful residues. For example, Zhao et al. (2006) successfully used two CE strains (lactic acid bacteria) to inhibit *L. monocytogenes* in floor drains at 3 to 26°C in a poultry processing plant. As many studies have concluded, microbial diversity is a key factor in avoiding pathogen outbreaks, and there is a need to search for highly effective CE microorganisms from environmental sources where *Listeria* reside. Animal waste–based composts are especially rich in microbial species that carry out various functions of pathogen inactivation, primarily due to heat generation, during the composting process, but the compost microbial community also possesses the suppressive activities against a variety of plant pathogens and some human pathogens (Jiang and Shepherd, 2009; Sidhu et al., 2001). For example, Sidhu et al. (2001) reported less than 3 log of *Salmonella* Typhimurium growth in non-sterilized compost as compared with more than an 8 log increase in sterilized biosolids, clearly indicating the importance of maintaining biologically active indigenous microflora to reduce the *Salmonella* regrowth potential in compost. Therefore, further investigation on the role of total microbial concentration and diversity in suppressing pathogen regrowth in biological soil amendments is needed.

Metagenomic and metatranscriptomic sequencing have become powerful tools for characterizing the structure, functional capabilities, and activities of complex microbial communities. Genomic techniques based on PCR amplification of the conserved and variable regions of the microbial genome (16S rRNA) allow direct sequencing of these PCR amplicons using high-throughput next-generation sequencing. The 16S rRNA sequencing has been used to characterize the microbial community structure in poultry litter (Smith et al., 2014). Using the same technology, Vida et al. (2016) discovered that increases in some orders of *Gammaproteobacteria*, *Betaproteobacteria*, and *Dothideomycetes* in agricultural soil amended with composted almond shells were responsible for suppressing the avocado soilborne phytopathogen, *Rosellinia necatrix*. However, a limitation of genomic DNA sequencing analysis is the inability to differentiate live cells (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 (Rogers et al., 2013). Therefore, high-throughput sequencing combined with PMA treatment can theoretically analyze DNA only in the living cells. 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. This advanced technique has successfully been used previously by Martins et al. (2013) to characterize the dynamics of microbial interactions and the role of microbial enzymes in degradation of biomass in compost. Their results demonstrated that biomass degradation during composting is fully performed by bacterial enzymes, possibly derived from *Clostridiales* and *Actinomycetales*. Analyzing the metatranscriptome is especially useful in examining populations that may be in flux, such as compost, which can be impacted and altered by a diverse array of transitory factors, such as moisture, temperature, O_2, microbial species, and so on. This is also complimentary, and adds an additional taxonomic signature to amplicon-based community profiling. To our knowledge, despite a few metagenomic studies of thermophilic composting processes, no studies have been carried out on the functional metatranscriptomics of human pathogen interactions with indigenous microorganisms in animal waste–based biological soil amendments.

Culture-based methods are essential for isolating microorganisms. Due to the presence of significant large populations of non-culturable microorganisms in natural environments and our limited knowledge of indigenous microbial species, less than 1% of the microorganisms present in the environment, such as soil or natural water samples, have been cultured with currently known methods. Recent attempts such as adding specific nutrients and substrates or

resuscitation promoting factors (Rpf), supernatants of growing cells, and modified incubation conditions have been investigated to resuscitate or recover those not previously cultivated microorganisms (Pinto et al., 2011). For example, Jin et al. (2017) successfully isolated viable but non-culturable (VBNC) bacteria from printing and dyeing wastewater bioreactors with the culture media containing Rpf protein secreted by *Micrococcus luteus*.

In this project, the combination of metatranscriptomics and culturing approaches should speed up the discovery of potential CE microorganisms with antagonistic activities against *L. monocytogenes* as this high-throughput sequencing technology can reveal a more comprehensive microbial community profile in compost. In addition to identifying CE species, large sequencing datasets generated by metagenomic approaches can be a great source to search for unique microbial species for plant disease suppression, environmental monitoring or discover novel microbial species (Antunes et al., 2016).

Research Methods and Results

Objective I*: Use of 16S rRNA and 18S rRNA sequencing to profile the microbial communities of biological soil amendments*

Compost sample collection: A total of 12 biological soil amendments (6 dairy and 6 poultry waste–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, we requested samples of both active compost (collected from the thermophilic composting stage [>55°C /131°F] within 1 month of composting) and finished compost, with the same ingredient. Using the sampling protocol recommended by the California Leafy Greens Marketing Agreement (LGMA), samples were collected in Ziploc bags, shipped under ambient condition to our lab, and stored at refrigeration conditions (4°C) once received. To reduce DNA degradation and microbial population change, the preparation and microbiological analysis for most samples were performed within 10 days of the samples being delivered.

Physical-chemical and microbiological analyses of biological soil amendments: Compost samples were analyzed for total aerobic bacterial count, *Actinomyces*, fungi, *Enterobacteriaceae*, and heterotrophic bacteria by plating a series of dilutions onto 3M™ Petrifilm™ aerobic count plates, Actinomycete Isolation Agar (AIA), Rose Bengal Agar (RBA), Violet Red Bile Glucose Agar (VRBG), and Reasoner's 2A agar (R2A), 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, and 25°C for 5–7 days, respectively. Samples were also examined for the presence of background *L. monocytogenes* by following the Bacteriological Analytical Manual (BAM; FDA) procedure. Moisture content was measured with a moisture analyzer (model IR-35, Denver Instrument, Denver, CO), and pH values were measured based on the methods described by Weatherburn (1967) and U.S. Composting Council (2002). Additionally, duplicate samples were analyzed for chemical characteristics, including total nitrogen, carbon, organic matter, and soluble salts (Table A1, Appendix A). The physical-chemical results along with the compost collection information (compost sources, collecting location, and composting stage) were used as metadata for microbial profiling and functional metatranscriptomic sequencing analysis.

Total culturable aerobic bacterial counts in the compost samples ranged from approximately 7.58 to 9.72 log CFU/g (Table A2, Appendix). For all categories, relatively higher levels of total aerobic bacteria and thermophiles were observed for the active compost as compared to the finished compost collected from the same farm. Yeast/mold counts ranged from <2 to 6.3 log CFU/g, and the population level of yeast/mold was found significantly ($P < 0.05$) lower than the

population of culturable bacterial species. After selective enrichment, some black colonies were observed on Oxford agar, but those black colonies were then purified and confirmed as non–*L. monocytogenes* with PCR targeting the *hlyA* gene.

Compost inoculation and L. monocytogenes *enumeration:* The experimental conditions were optimized using denaturing gradient gel electrophoresis (DGGE). As shown in Figure A1 (Appendix A), band patterns of *L. monocytogenes* in compost samples were visible at both 7 and 9 log inoculation levels at 72 h of incubation, but the DGGE pattern of compost samples with 9 log *L. monocytogenes* was distinctly different from that of compost samples with the lower inoculation level. Therefore, the results from DGGE indicated that *L. monocytogenes* should be inoculated at 7 log CFU/g or higher and incubated for 72 h in compost before sequencing analysis.

Based on the results described above, the optimized experimental conditions for the full-scale sequencing analysis were selected (Figure A2, Appendix). The thoroughly mixed compost samples were adjusted to 40 and 80% moisture content and then inoculated with *L. monocytogenes* strain FSL R9-5506 (a pathogenic strain isolated from packaged salad, kindly provided by Dr. Martin Wiedmann at Cornell University) or not. To prepare the inoculum, the overnight grown *L. monocytogenes* was washed and resuspended in 0.85% saline to ca. 10^9 CFU/ml. Afterwards, the culture was inoculated into compost samples with the target moisture contents at a final inoculation level of ca. 7 log CFU/g. At 0 and 72 h incubation, *L. monocytogenes* populations in the compost samples were enumerated by plating onto Oxford agar, followed by incubation at 35°C for 24 h. Overall, 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 1**), whereas more than 0.5 log CFU/g of regrowth occurred in the compost samples with 80% moisture content from poultry farm #2 and dairy farm #1.

DNA extraction and initial quality control (QC) of extracted DNA: After 0 and 72 h incubation at room temperature, the samples were treated with propidium monoazide (PMA) prior to DNA extraction to avoid the extraction of nucleic acids from dead cells. Briefly, 50 µM PMA was incubated with the compost slurry (1:4, w/v) in the dark, and then cross-linked under a 650 W halogen light source. The genomic DNA (gDNA) was extracted by DNA isolation kit (ZymoBIOMICS DNA miniprep). In total, 288 gDNA samples were extracted for Obj. I (Figure A2, Appendix). The quality of extracted gDNA was checked by Nanodrop 2000 and Qubit, and no degradation of gDNA or RNA contamination was detected by gel electrophoresis. Based on the level of viable yeast/mold counts and the population reduction of *L. monocytogenes*, we decided to select only the gDNA samples extracted from poultry farm #1 and dairy farm #2 composts for 18S rRNA sequencing. In total, 288 and 96 gDNA samples were submitted to ZymoBIOMICS Services for bacterial 16S rRNA and fungal 18S rRNA targeted sequencing, respectively.

Targeted library preparation and sequencing: Bacterial 16S ribosomal RNA gene targeted sequencing was performed using the Quick-16S NGS library prep kit (Zymo Research, Irvine, CA). The bacterial 16S primers were used to amplify the V3-V4 region of the 16S rRNA gene. Fungal 18S gene target sequencing was performed with custom 18S primers (Euk1391f-EukBr). These primers have been custom-designed by Zymo Research to provide the best coverage of the 16S and 18S genes while maintaining high sensitivity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator, then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit. The final library was sequenced on Illumina MiSeq with a V3 reagent kit (600 cycles). The sequencing was performed with >10% PhiX spike-in (a concentrated Illumina control v3 library).

Sequencing data processing: Unique amplicon sequences were inferred from raw reads and chimeric sequences were also removed using the DADA2 pipeline (Callahan et al, 2016). Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research database. Before further analysis, the raw counts were adjusted for sequencing depth by even sampling and for 16S copy number variation using copy number estimates from the Ribosomal RNA Database (rrnDB) v5.4.

Summary of tools and findings from bioinformatic and multivariate statistical analysis: **Table 2** summarizes the bioinformatic and multivariate statistical tools and results. In summary, over 800 indigenous bacterial genera and 180 fungal species were identified from compost samples by 16S and 18S targeted sequencing, respectively. Most of these genera observed in either one or more compost types studied here were like those reported earlier in other studies, including bacteria from the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. The compost source, composting stage, and geographic location of the composting facility showed significant effects on the microbial diversities within each compost sample and on the microbial compositions between samples. As for the experimental variables, the effect of incubation time was much stronger than that of compost moisture or *Listeria* inoculation. Through plot visualization, the microbial community of samples with *Listeria* inoculation does separate from those without inoculation within the same sample group, i.e. sample collected from the same composting stage on the same farm. **Bacterial genera including *Bacillus*, *Sphaerobacte*r, *Pseudomonas*, *Brumimicrobium*, *Flavobacterium*, *Filomicrobium*, *Altererythrobacter*, and *Steroidobacter* were identified as potential CE microbial members that separate *L. monocytogenes*–inoculated and uninoculated communities from multiple farms**.

Investigating taxonomic compositions of poultry and dairy composts using phylogenetic marker analysis based on the sequencing of 16S for prokaryotes and 18S for eukaryotes: Four types of compost, including compost samples from two sources (dairy or poultry), and compost samples from two composting stages (active and finished), were studied in this project. 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 all operational taxonomic units (OTUs) of all compost samples. In addition, *Nucletmycea Fungi*, *Cercomonadidae Cercomonas*, *Arachnida Acari*, *Chromadorea Rhabditida*, and *Chromulina cf. nebulosi* were the top-5 dominant fungal species found in composts collected from poultry farm #1 and dairy farm #2.

The microbial community as revealed by 16S targeted sequencing was further studied; to get an overall view on the microbial distribution of key genus in compost samples from different farms, clustered heatmaps were constructed for dairy and poultry compost (Figure A3, Appendix). The heatmap plot depicts the relative percentage of the key bacterial genera (making up more than 5% of total composition in dairy or poultry compost, respectively; variables clustering on the X-axis) within each sample (Y-axis clustering). Clearly, the composting stage and location had significant impacts on the microbial compositions of both dairy and poultry compost samples. In agreement with heatmap visualization, the distribution of microbial communities was separated well between active and finished composts across all farms; for example, the bacterial community composition in dairy farm #1 composts is shown in Figure A4 (Appendix). Nonetheless, the discriminatory taxa contributing to the variation in microbial communities among different compost types (active dairy compost, finished dairy compost, active poultry compost, and finished poultry compost) need to be studied as well.

At the phyla level, the relative abundance of each phylum representing >1% of the total OTU is shown in Figure A5-A (Appendix). *Chloroflexi* represented a significantly greater proportion of

reads in finished dairy compost compared with other types. Regardless of the compost source, compost at the thermophilic phase (active compost) showed much lower relative abundance of *Acidobacteria* but higher abundance of *Bacteroidetes* as compared with the finished compost. Further, linear discriminant analysis (LDA) effect size (LEfSe) was used to detect microbial genus differentiating among different compost types. As shown in Figure A5-B, LEfSe results indicated that those discriminatory microbial species at the genus level were more harbored in active dairy compost (18 genera) than in the other three compost types, i.e. 10 genera for finished dairy and active poultry composts, respectively, and 12 genera for finished poultry compost. **At the genus level, 15 genera used as biological control agents for preventing plant or soil pathogens were identified from our compost samples by 16S targeted sequencing (**Table A3, Appendix**).** As compared to other farms, the potential biological control genera make up more than 18% of total genera identified in compost from dairy farm #1.

Factors affecting the microbial structure and diversity within each compost sample: Alpha diversity indices, including Shannon, Inverse Simpson index, species richness estimator of Chao 1, and observed species, were used to measure the community diversity of each sample. Rarefaction curves were created by plotting the alpha diversity measurement at different sequencing sizes. As shown in Figure A6 (Appendix), the curves all reached a plateau, indicating that the sequencing depth for all samples was sufficient to cover compost microbial community diversity. Non-parametric Kruskal-Wallis tests were performed to test factors that affect the alpha diversities. As shown in Table A4 (Appendix), all the compost related factors have significant effects on the Shannon and Inverse Simpson diversities, whereas only C:N, composting stage, and collection location significantly affected the Chao richness and observed species. **Among the experimental variables, all the alpha diversity indices for all compost samples were significantly affected by moisture content and incubation time overall, but not by *Listeria* inoculation**.

With regard to the impact of *Listeria* inoculation on the alpha diversities on individual farms, the introduction of *L. monocytogenes* affected the Chao richness and Observed species indexes of the finished compost samples collected from poultry farm #1 ($P < 0.05$; Figure A7, Appendix). These results showed the species richness of this compost increased after *L. monocytogenes* inoculation, indicating that compost samples collected from poultry farm #1 can be a candidate source for investigating CE microorganisms against *L. monocytogenes* for Obj. III.

Variation in microbial compositions of composts among different treatments (β-diversity):
Exploratory principle-coordinate analysis (PCoA) using a phylogenetically defined weighted UniFrac distance matrix was performed to visualize the differences between compost samples. Samples were distributed in the ordination space based largely on their group identities (collection location, compost type or composting stage, experimental moisture, incubation time, presence or absence of *L. monocytogenes*). Figure A8 (Appendix) shows PCoA results based on the analysis of the genus abundance data set for dairy (A) and poultry (B) composts. **Although the microbial community of the compost samples was separated by compost type and farm, samples with *Listeria* inoculation do separate from those without, albeit within each sample group.**

Further, the significance of different factors was tested by analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA). For both analyses, as shown in Table A5 (Appendix), compost source (dairy or poultry), composting stage (active or finished), and collection location have significant effects on the microbial community composition in compost samples, with P-values < 0.001. Incubation time was found to be a significant experimental factor by both ANOSIM and PERMANOVA, with P-values < 0.05.

In addition, the effects of experimental variables were visualized by constrained ordination analysis using canonical correspondence analysis (CCA) using three experimental variables, i.e. experimental moisture (40% vs 80%), incubation time (0 h vs 72 h), and *L. monocytogenes* (0 log CFU/g vs 7 log CFU/g), as explanatory variables. The relationships between explanatory variables and microbial compositions were represented by the length and angles of red arrows. Overall, results from CCA suggested that the impact due to *Listeria* inoculation was not as strong as experimental moisture and incubation time on the microbial community composition (Figure A8-C, Appendix), which agrees with results from PERMANOVA and ANOSIM. Moreover, the microbial compositions of compost samples from dairy farms #1 and #3, and poultry farm #2 were significantly affected by both incubation time and experimental moisture ($P < 0.05$), whereas the microbial composition of compost samples from poultry farm #3 was only affected by incubation time ($P < 0.05$) (Table A6, Appendix).

Lastly, SIMPER was used to identify the microbial members that separate inoculated and uninoculated communities in different composts. The differences in the relative abundance of these discriminant genera in dairy and poultry composts are shown in Figure A9-A&B (Appendix). As a result, *Sphaerobacter*, *Bacillus*, *Pseudomonas*, *Brumimicrobium*, *Flavobacterium*, *Filomicrobium*, *Altererythrobacter*, and *Steroidobacter* were identified as top microbial members that separate *L. monocytogenes*–inoculated and uninoculated communities observed in multiple farms, indicating there was a higher chance to isolate CE strains by culturing methods from these genera. Moreover, those potential CE microorganisms were highly abundant in both active and finished composts from dairy farm #1, and active compost from poultry farm #3, therefore, those compost samples can be candidate sources for isolating CE strains against *L. monocytogenes* in Obj. III (**Figure 1**). It needs to be pointed out that the **interactions between these discriminatory genera and *L. monocytogenes* were found to vary in different composts, indicating that the microbial interactions were influenced by their habitable environments**. Also, the top discriminatory microbial members that increased in relative abundance after *L. monocytogenes* inoculation were also identified from each farm (Table A7, Appendix).

***Objective II**: Functional metatranscriptomic analysis of* L. monocytogenes *interactions with indigenous compost microflora to identify CE microorganisms for antagonistic activity against the pathogen*

Based on the results from Obj. I, the active compost collected from dairy farm #1 was selected for Obj. II because there were more potential CE microorganisms identified in compost samples collected from dairy farm #1 compared with the other farms.

Compost sample collection and study design: To prevent RNA degradation prior to analysis, fresh active compost was requested from dairy farm #1. Two collections were performed from two composting rows with the same ingredients and composting length to serve as biological replicates. Immediately after receiving the compost samples, *L. monocytogenes* strain FSL R9-5506 was inoculated into compost samples with 80% moisture content, as described in Obj. I. Total RNA was extracted from compost samples immediately after incubation for 0 and 72 h with or without *L. monocytogenes* by using the ZymoBIOMICS DNA/RNA extraction kit. The extracted total RNA was further purified using the RNA Clean & Concentrator kit. RNA integrity number (RIN) was determined using RNA 6000 Nano kit by the 2100 Bioanalyzer, and RNA concentration was measured by Qubit. After QC steps for the extracted RNA, although RIN scores of our RNA samples were all >7, the total yield of RNA extracted from compost samples was lower than the amount required by the sequencing facility (Novogene Inc.). As a result, we decided to pool the multiple extractions to increase the RNA yield. To analyze community

functional capacity of indigenous compost microorganisms in the presence of *L. monocytogenes*, shotgun metagenomic sequencing was also employed for Obj. II.

Library construction and quality control (QC), and sequencing: Both DNA and RNA samples were submitted to Novogene Inc. for metagenomic and metatranscriptomic sequencing, respectively. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit 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. rRNA was removed using the Ribo-Zero kit, and the mRNA was concentrated. Following the QC steps, the qualified libraries from DNA and RNA were fed into Illumina sequencers after pooling according to its effective concentration and expected data volume, respectively.

Sequencing data QC and Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) analysis: To get clean reads for both metagenomic and metatranscriptomic sequencing, reads containing adapter and low-quality base (Qscore ≤5) were removed from the raw reads, followed by a further filtering and trimming step by AfterQC (a tool for automatic filtering, trimming, error removing, and quality control for fastq data). All the metagenomic sequencing data were having >99% good reads after filtering, and for the metatranscriptomic sequencing, all samples have a >6 G output, with adapter rate <10%. After these steps, all reads were uploaded into the MG-RAST analysis server, which is an open source service made up of a high-throughput pipeline built for high-performance computing of metagenomes. 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 analysis: Statistical comparisons of the proportions of functions among samples or among groups of samples were conducted in STAMP software (Statistical Analysis of Metagenomic Profiles; Parks et al., 2014). Briefly, 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 to compare two groups of samples, and Welch's inverted method was to calculate 95% confidence intervals.

Microbial community composition and functional capacities of compost as revealed by shotgun metagenomic sequencing analysis: The analysis of the taxonomic community showed that 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%). As compared with the results from Obj. I, shotgun metagenomic and 16S rRNA targeted sequencing results agree with each other. **The five most abundant phyla in active dairy compost collected from dairy farm #1 were *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes*,** and these five phyla account for at least 91% of all classified reads in the active dairy compost samples.

The metagenomic sequencing provided insights into the metabolic potential of organisms inhabiting the collected compost samples. The predicted proteins were annotated using the SEED subsystems (Level 1) firstly. As shown in Figure B1 (Appendix), carbohydrates, clustering-based subsystems, and amino acids and derivatives dominated in the active dairy compost samples. Among these dominant level 1 subsystems, the major level 2 subsystems in carbohydrate were one-carbon metabolism and organic acid. These results are consistent with

the taxonomic analysis, in which the dominant abundances of *Firmicutes* and *Actinobacteria* were observed and these bacterial groups directly contribute to the carbohydrate metabolism by fermentation or biomass degradation. In summary, this observation confirmed that **our results based on the DNA shotgun sequencing data reflect actual microbial functional capacities in the active dairy compost.**

To gain a comprehensive understanding of the functional capacities of active dairy compost from farm #1, two separate 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 significantly different ($P < 0.05$) between the two collections (Figure B2, Appendix). For example, the functional profiles associated with DNA metabolism and motility and chemotaxis were found to be higher in relative proportions in collection A than in collection B, whereas fatty acids, lipids, and isoprenoids were higher in relative proportions in collection B. Presumably, the variance in functional profiles of these two collections may be due to the heterogeneous mixtures of compost samples.

Comparison of the microbial functional capacities with and without L. monocytogenes *by metagenomic sequencing analysis:* At the SEED subsystem level 2, 13 of 196 pathways were significantly different in the compost samples in the two collections ($P < 0.05$), suggesting the means of proportions of these pathways from all treatment groups (compost sample inoculated with or without *L. monocytogenes* at 0 or 72 h incubation periods) are not equal. Next, we used 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 ($P < 0.05$). These pathways include bacteriocins, ribosomally synthesized antibacterial peptides, phage and prophages, catabolism of an unknown compound, and monosaccharides #1 etc. (highlighted in bold in Table B1, Appendix B). 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 ($P \geq 0.1$). **Clearly, sufficient contact time is required to allow the pathogen (*L. monocytogenes*) to interact with indigenous microorganisms and to induce the change in microbial functional capacities.** Besides, the increased abundance in the bacteriocins category suggested that the active compost sample may contain certain microbial species that are active against the invasion bacteria by producing such ribosomally synthesized antimicrobial peptides.

As mentioned, the microbial functional capacities from compost samples varied between the two collections. Therefore, at 72 h incubation, the microbial functional profiles of composts with and without inoculation of *L. monocytogenes* as classified at SEED functional gene entries were further analysed separately for the two collections. 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 ($P < 0.05$) enriched in compost samples inoculated with *L. monocytogenes*, whereas meiosis-specific DNA cleavage protein was found in more abundance in proportions in the compost samples without *L. monocytogenes*. These observations were consistent among the technical replicated samples in collection B (Figure B3, Appendix). 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 above mentioned 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, as mentioned previously.

Identification of gene expression changes associated with specific microbial functional pathways in compost with and without L. monocytogenes *by metatranscriptomic sequencing analysis:* Similar to the metagenomic sequencing analysis, multiple groups 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 197 pathways of active functional categories were found at the SEED subsystem level 2 from multiple group comparison. Only selected categories with $P < 0.001$ are presented in Table B2 (Appendix). The impact of *L. monocytogenes* on gene expression level associated with specific functional pathways of compost microflora was assessed by metatranscriptomic sequencing analysis after 72 h of incubation period. Based on the pairwise testing of the total functional genes annotated with the KEGG database, 117 of the 4414 functional genes were significantly differentially expressed between compost samples with and without *L. monocytogenes* inoculation, given a *P*-value sufficiently small ($P < 0.05$), and 56 of these 117 genes were categorized as metabolism at level 1 in the KEGG database, 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 2**). As previously observed in the metagenomic analysis, the changes in the metabolic pathways in compost samples containing *L. monocytogenes* indicated that the interactions between *L. monocytogenes* and competitive exclusion bacteria may include the competition for compost nutrients. This observation can explain one of the conclusions from Obj. I, that the microbial interactions were influenced by their habitable environments due to the different nutrients provided by different types of compost.

In addition to competing for nutrients, the production of bacteriocins by microorganisms was another mechanism for *L. monocytogenes* reduction. As revealed by metagenomic sequencing, abundance in the bacteriocins category increased in the compost samples inoculated with *L. monocytogenes* after 72 h (Figure B4-A, Appendix). Results from metatranscriptomic sequencing also showed that the mean proportions of microbial functions associated with bacteriocins, ribosomally synthesized antibacterial peptides, significantly ($P < 0.05$) increased in the compost sample with *L. monocytogenes* after 72 h incubation, with a higher mean proportion (Figure B4-B, Appendix). A similar trend was observed for the function associated with phages, prophages (Figure B5, Appendix). **As compared to metagenomic sequencing, metatranscriptomic sequencing can provide the active gene expression levels associated with bacteriocin production, and therefore can be used to monitor how these levels change when *L. monocytogenes* is present in the compost.**

Based on Obj. I and II results, although the experimental design varied among three approaches due to the project budget and time limit, metagenomics sequencing is preferred if research focus is 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 a complex matrix, the large-scale application of metatranscriptomic sequencing of compost samples is limited currently. Importantly, in order to understand the complex microflora in compost, more biological replicates should be collected for any sequencing approach.

Objective III: *Optimizing culturing conditions to isolate and validate CE microorganisms with antagonistic activities against* L. monocytogenes

*Screening potential competitive exclusion (CE) strains for anti-*Listeria *from biological soil amendments using the double-agar-layer method*: All the biological soil amendments collected

from Obj. I were used for initial screening of CE candidate strains by the overlay method. Serial dilutions of the compost samples were made with sterile saline (0.85%, w/v NaCl), and the proper dilutions were plated onto Tryptic Soy Agar (TSA) and incubated at 35°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 10^7 CFU/ml of *L. monocytogenes* strains FSL R9-5506, Scott A, and LCDC, respectively. All the plates were incubated at 35°C for 24 h, and colonies with the growth inhibition zone were isolated and purified. The antagonistic activity against *L. monocytogenes* by the CE candidate strains was further confirmed by the agar-spot-test method. Briefly, the CE isolates were spot-inoculated onto the TSA plates. After overnight incubation, 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, respectively. The zone-of-inhibition was observed after overnight incubation at 37°C (Figure C1, Appendix C).

Screening potential competitive exclusion (CE) strains for anti-Listeria *from biological soil amendments by modified agar plates using triple-agar-layer method*: Based on the results of Obj. I, compost samples with high abundance of the potential CE microorganisms, namely both active and finished composts from dairy farm #1 and active compost from poultry farm #3, were selected for Obj. III. Additionally, as a relatively higher level of culturable bacterial population was observed for the active compost as compared to the finished compost collected from the same farm, active composts from other farms were also used to isolate CE microorganisms against *L. monocytogenes*.

Two types of modified agar plates, i.e. MPN + resuscitation promoting factor (Rpf) and PYG, were used in an effort to grow the viable but non-culturable bacteria. 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). *Micrococcus luteus* ATCC 4698 was used to prepare Rpf. *M. luteus* was inoculated to the LMM medium (containing 5 g peptone, 3 g yeast extract and 1 g $MgSO_4 \cdot 7H_2O$, pH 7.0 per liter). The medium was shaken at 120 rpm and incubated at 30°C for 36 h. The bacterial culture was then inoculated to new LMM medium (4%, v/v) and incubated at 30°C for another 60 h according to Jin et al. (2017). The fermentation broth was centrifuged, and then filtered through a 0.22 µm filter to remove floating cells. Finally, the supernatant containing Rpf was obtained, stored at −20°C, and used as supplementary material for media preparation.

The proteose-yeast-glucose (PYG) constituents of the medium were grouped into three solutions (solutions A, B, and C). Solution A contained basal salts and Bacto agar (final concentration 15 g/L), solution B was phosphate buffer (final concentration 20 mM phosphate [pH 7]), and solution C contained Bacto peptone, Bacto yeast extract, and glucose, with final concentrations of 0.1 g/L each. Using these solutions, agar media was prepared by separately autoclaving phosphate and agar (PS) protocols: solutions A, B, and C were separately autoclaved and subsequently mixed (Tanaka et al. 2019).

Serial dilutions of the compost samples were made with sterile saline (0.85%, w/v NaCl), 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. 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 more than 7 days after the start 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 10^7 CFU/ml of *L. monocytogenes* strain FSL R9-5506, Scott A, and LCDC, respectively. Colonies with inhibition zone were picked up, followed by transfer to fresh TSA for further purification. The

purified colonies from the previous step were subjected to testing the antagonistic activity against *L. monocytogenes* by spot-on-lawn methods as described above.

The number of new colonies cultured by PYG agar plates increased after 14 days of incubation, indicating the presence of slow-growing bacteria in compost samples (Table C1, Appendix). Through different culturing methods**,** a total of 40 isolates (15 from poultry compost and 25 from dairy compost) were isolated and confirmed with anti–*L. monocytogenes* activities. MPN + Rpf can be used to isolate the microorganisms with low abundance in compost samples, such as *Kocuria* spp. (<0.03% of relative abundance). These CE isolates have been identified as *Bacillus* spp. (20), *Kocuria* spp. (12), *Paenibacillus* spp. (4), *Brevibacillus* spp. (1), and *Planococcus* spp. (3) by Sanger sequencing. In agreement with findings from Obj. I, 50% of the isolated CE strains by culturing methods were identified as *Bacillus*. These 40 CE isolates were then separated into two groups, i.e. poultry CE group and dairy CE group, for co-culturing studies against *L. monocytogenes* in compost extract models. The detailed species identifications for these CE strains are listed in Table C2 (Appendix). Note that some CE species have been already used as biological control agents for crops, including potato, soybean, and strawberry.

Application of CE microorganisms to inhibit growth of L. monocytogenes *in compost extracts:*
Dairy and poultry composts were extracted with sterile tap water at different ratios (1:5 and 1:10, w/v) in a shaking incubator (100 rpm, 22°C) for 24 h, and centrifuged at 5,000 xg for 20 min. The sterile supernatants were collected by filtering through a 0.2-µm filter, and then stored at −20°C until use. Both dairy and poultry CE groups were tested in dairy and poultry extracts, respectively. Briefly, the compost extract inoculated with a cocktail of rifampicin-resistant *L. monocytogenes* strains (R9-5506, Scott A, and 101M) (ca. 3 log CFU/ml), was inoculated with or without ca. 7 log CFU/ml of CE strains and incubated at room temperature or 35°C for up to 7 days. At selected intervals (0, 24, 72, 120, and 168 h), the samples were serially diluted and plated onto TSA supplemented with rifampicin (100 µg/ml) and onto regular TSA for enumeration of *Listeria* and CE cells, respectively.

During the growth inhibition study (Figures C2 & C3, Appendix), no change ($P > 0.05$) was observed for CE populations in poultry compost extracts, but CE populations increased by 0.3 to 0.6 log in the dairy compost extracts. After 168 h incubation, reductions of *L. monocytogenes* in poultry compost extracts with extraction ratios of 1:5 and 1:10 were 1.1 and 1.1 log at 35°C or 0.9 and 0.7 log at room temperature. In contrast, after 24 h incubation, the reductions of *L. monocytogenes* in dairy compost extracts with extraction ratios of 1:5 and 1:10 were 2.2 and 1.7 log at 35°C or 1.6 and 1.5 log at room temperature (**Table 3**). **Clearly, the growth of *L. monocytogenes* was 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 increased in the more concentrated dairy compost extracts (1:5) at 35°C and room temperature**.

Outcomes and Accomplishments

Objective I*: Use of 16S rRNA and 18S rRNA sequencing to profile the microbial communities of biological soil amendments*

The team obtained 12 different biological soil amendments (composts) for this project, and compared the culturable microorganisms and the reductions of *L. monocytogenes* among these composts. Bioinformatic and multivariate statistical analysis tools, including QIIME, R program, PAST3, and online Galaxy were used for data analysis. Through targeted sequencing, the major

bacterial phyla from all collected compost samples were identified as *Firmicutes* (23%), *Proteobacteria* (23%), *Actinobacteria* (19%), *Chloroflexi* (13%), *Bacteroidetes* (12%), *Gemmatimonadetes* (2%) and *Acidobacteria* (2%), accounting for 94% of all OTUs of all compost samples. In addition, *Nucletmycea Fungi*, *Cercomonadidae Cercomonas*, *Arachnida Acari*, *Chromadorea Rhabditida*, and *Chromulina Chromulina cf. nebulosi* were the top-5 dominant fungal species found in composts collected from poultry farm #1 and dairy farm #2.

The microbial richness and diversity within composts varied among samples and were significantly affected by the compost source, compost stage, and collection farm. Sequencing data for microbial compositions of compost samples with or without *L. monocytogenes* inoculation were extensively analyzed. Results indicated that the microbial community of the samples was separated by compost types and farms in exploratory ordination analysis, and samples with *Listeria* inoculation did separate from those without, albeit within each sample group. In general, active dairy compost is rich in the identified potential CE species.

Objective II: *Functional metatranscriptomic analysis of L. monocytogenes interactions with indigenous compost microorganisms to identify CE microorganisms for antagonistic activity against the pathogen*

The analysis of the taxonomic community showed that active compost from dairy farm #1 was dominated by bacteria (98.14%), followed by archaea (0.97%), eukaryote (0.53%), virus (0.01%), and other unassigned sequences (<0.01%). In agreement with Obj. I findings, the five most abundant phyla in compost from dairy farm #1 were *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes*. For metagenomic sequencing, some functional capacities increased in mean proportions due to inoculation of *L. monocytogenes* after 72 h incubation ($P < 0.05$), but not at 0 h post inoculation, indicating sufficient contact time is required to allow the pathogen (*L. monocytogenes*) to interact with indigenous microorganisms and to induce the change in microbial functional capacities. As compared to the metagenomic sequencing, metatranscriptomic sequencing revealed more active functional categories (75 of 197 pathways) at the SEED subsystem level 2 among different treatment groups. Results from both sequencing approaches indicated that the interactions between *L. monocytogenes* and compost microflora may include the competition for compost nutrients and the production of bacteriocins by CE bacteria.

Objective III: *Optimizing culturing conditions to isolate and validate CE microorganisms with antagonistic activities against* L. monocytogenes*.*

For Obj. III, through different culturing methods**,** a total of 40 isolates (15 from poultry compost and 25 from dairy compost) were confirmed with anti–*L. monocytogenes* activities. MPN + Rpf was effective for isolating the microorganisms with low abundance in compost samples, such as *Kocuria* spp. (<0.03%). The 40 CE isolates have been identified as *Bacillus* spp. (20), *Kocuria* spp. (12), *Paenibacillus* spp. (4), *Brevibacillus* spp. (1), and *Planococcus* spp. (3) by Sanger sequencing. In agreement with our bioinformatic findings in Obj. I, 50% of the CE strains isolated by culturing methods were identified as *Bacillus*. Moreover, 10 species and 1 genus from these CE members have been reported as biological control agents for crops, including potato, soybean, and strawberry. We also evaluated these CE strains against three strains of *L. monocytogenes* using a growth inhibition study. In the presence of ca. 7 log CE, the growth of *L. monocytogenes* was significantly inhibited in both concentrated poultry and dairy compost extracts at 35°C, whereas more than 1.4 log reductions occurred in dairy compost extracts after 24 h incubation.

Summary of Findings and Recommendations

***Objective I**: Use of 16S rRNA and 18S rRNA sequencing to profile the microbial communities of biological soil amendments*

A full bioinformatics pipeline was optimized in this project to analyze the microbial community structure of biological soil amendments in the presence and absence of *L. monocytogenes* based on the targeted sequencing. The microbial communities of compost samples were separated based on both the compost source and composting stage. Overall, from the compost samples the team found 15 genera that have been identified as potential biological control agents for controlling plant or soil pathogens, suggesting that composts are rich sources for potential CE discovery. Results from discriminant analysis showed that the abundance of some microbial members changed due to the addition of *L. monocytogenes*, implying that there is a greater chance to isolate CE strains from these discriminant genera by culturing methods. This finding was later confirmed in Obj. III, where 50% of the CE strains isolated by culturing methods were identified as *Bacillus*, suggesting the advanced sequencing approach can be a powerful tool for selecting the potential biological control genera (CE strains) from biological soil amendments.

***Objective II:** Functional metatranscriptomic analysis of L. monocytogenes interactions with indigenous compost microorganisms to identify CE microorganisms for antagonistic activity against the pathogen*

To our knowledge the methods and data from this compost analysis project are reported for the first time, and can be very useful for the composting industry to better understand the microbial community and functionality in their products, and to discover CE microorganisms more efficiently. Based on our sequencing results, the increased abundance in bacteriocins upon the introduction of *L. monocytogenes* suggests that the active compost may contain certain bacterial species that are antagonistic against the invasion pathogen by producing ribosomally synthesized antimicrobial peptides. Furthermore, the changes in the metabolic pathways in compost samples containing *L. monocytogenes* indicated that the interactions between *L. monocytogenes* and compost microflora may include competition for compost nutrients. Overall, as compared to the metagenomic sequencing, metatranscriptomic sequencing can provide the active gene expression levels associated with bacteriocin production, and therefore can be used to monitor how these levels change when *L. monocytogenes* is present in the compost.

***Objective III:** Optimizing culturing conditions to isolate and validate CE microorganisms with antagonistic activities against* L. monocytogenes

Our research demonstrated that the modified double/triple-agar-layer procedure is a quick and efficient method for screening CE candidates from different biological soil amendments, and that the addition of Rpf promotes the growth of slow-growing/viable but non-culturable species. The growth inhibition of *L. monocytogenes* by CE strains in both dairy and poultry compost extracts was clearly demonstrated at all incubation conditions. Our research findings suggest that adding specific CE microorganisms into composts is a potential approach to inhibit the growth of *L. monocytogenes,* and a cocktail of CE microorganisms may be applied a few days prior to agricultural land application of the finished compost. We believe that those identified CE microorganisms can be used in biological control of *L. monocytogenes* in various ecosystems.

APPENDICES

Publications (*in preparation**)
Hongye Wang, Vijay Shankar, and Xiuping Jiang. Analyze microbial community structure of dairy and poultry compost using phylogenetic marker analysis based on the sequencing of 16S rRNA for bacteria in the presence and absence of *Listeria monocytogenes.* (*in preparation*)

Presentations (posters*)
Hongye Wang, Vijay Shankar, Muthu Dharmasena, and Xiuping Jiang. 2019. Analyzing microbial community change of turkey litter compost due to heat exposure using 16S high throughput sequencing [P1-266]. IAFP Annual Meeting, July 21–24, Louisville, KY.

Hongye Wang and Xiuping Jiang. 2020. Application of competitive exclusion microorganisms to inhibit the growth of *Listeria monocytogenes* in compost extract. IAFP Annual Meeting, October 25–28, Cleveland, OH. (*to be presented*)
* *Abstracts are available at the end of this report (pp.27–28).*

Budget Summary
The total funds awarded to this project were $48,523, and the team expects to use all funds.

The funds awarded were used strategically to complete the proposed objectives by reaching out to the sequencing companies for a deep discount on their services and prioritizing the samples being sequenced. However, additional funds would have allowed us to generate more comprehensive data.

Suggestions to CPS
We enjoyed the close contact with CPS, and all the activities, such as attending research symposiums and making industry contacts, which helps us to refine our research approaches in order to develop effective solutions for the produce industry.

Tables 1–3 and Figures 1–2: see below
(Appendix Tables and Figures are available in: **CPS Final Report - Jiang APPENDIX**)

Table 1 Population change of *L. monocytogenes* in biological soil amendments after 72 h incubation at room temperature

Collection farm	Compost type	Population change of *L. monocytogenes* in the compost with different moisture content (MC) (log CFU/g)	
		40% MC	80% MC
Poultry farm #1	Active compost	-0.37 ± 0.08	-0.12 ± 0.17
	Finished compost	-0.51 ± 0.19	-0.75 ± 0.07
Poultry farm #2	Active compost	-0.06 ± 0.33	0.52 ± 0.07
	Finished compost	-0.60 ± 0.28	1.54 ± 0.22
Poultry farm #3	Active compost	-0.28 ± 0.15	-0.41 ± 0.07
	Finished compost	0.07 ± 0.02	-0.77 ± 0.26
Dairy farm #1	Active compost	0.13 ± 0.02	0.72 ± 0.21
	Finished compost	0.14 ± 0.19	0.52 ± 0.35
Dairy farm #2	Active compost	-0.25 ± 0.29	-0.43 ± 0.16
	Finished compost	-0.58 ± 0.16	-0.72 ± 0.07
Dairy farm #3	Active compost	0.27 ± 0.11	-0.31 ± 0.27
	Finished compost	-1.05 ± 0.23	-0.79 ± 0.24

Table 2 Summary of bioinformatic and multivariate statistical analysis of 16S and 18S rRNA targeted sequencing data

Community data & Definition	Methods to assess community data	Software/tools	Functions	Major findings in this study	Figure or Table in Appendix A
Taxonomic distributions: Show the general characteristics of microbial compositions.	Heatmap visualization	R package heatmaply	Visualization of taxonomic clustering patterns among different groups.	The microbial compositions of compost were well clustered within composting stages and locations.	Fig. A3
	Bar graphs of taxa	R package phyloseq	Observation of taxonomic distributions.	Over 800 indigenous bacterial genera and 180 fungal species were identified from compost samples.	Fig. A4
	Linear discriminant analysis (LDA) effect size (LEfSe)	Online Galaxy	To find discriminatory taxa at different levels for each group.	The discriminatory species in the genus level were more harbored in active dairy compost as compared in the other three compost types.	Fig. A5
Alpha diversity: Use different indices to show species richness and diversity detected in a microbial ecosystem.	Bar/box plots	R package vegan	Composed from alpha diversity indices to visualize the differences.	Species richness of finished compost (poultry farm #1) sample increased after *L. monocytogenes* inoculation.	Fig. A7
	Kruskal-Wallis analysis	R package vegan	For testing the significance of differences for nonparametric data.	The compost related factors, moisture contents, and incubation time have significant effects on the Shannon and Inverse Simpson diversities.	Table A4

Table 2 *cont*. Summary of bioinformatic and multivariate statistical analysis of 16S and 18S rRNA targeted sequencing data

Community data & Definition	**Methods to assess community data**	**Software/tools**	**Functions**	**Major findings in this study**	**Figure or Table in Appendix A**
Beta diversity: Shows differences between microbial communities from different environments.	Principle coordinate analysis (PCoA) using weighted UniFrac metrics	Qiime v.1.9	Visualize the differences between samples using ordination plots with weighted UniFrac metrics.	The microbial community of samples was separated by compost type and farms, samples with *Listeria* inoculation do separate from those without, albeit within each sample group.	Fig. A8-A
Main focus is on the difference in taxonomic.	Analysis of similarity (ANOSIM) & Permutational multivariate analysis of variance (PERMANOVA)	R package vegan	Test the significance of differences for category factors & Test the significance of differences for both category and continuous factors.	For the experimental variables, incubation time was found to be significant experimental factors by both ANOSIM and PERMANOVA.	Table A5
	Canonical correspondence analysis (CCA)	PAST3	Constrained ordination analysis to find which experimental variables have the largest impact on microbial community composition.	The impact due to *Listeria* inoculation was not as strong as experimental moisture and incubation time on the microbial community composition.	Fig. A8-B & Table A6
	Similarity percentage (SIMPER)	R package vegan	Screening top taxa driving variation in microbial community composition.	Microbial members that separate inoculated and uninoculated communities in different compost were identified.	Fig. A9 & Table A7

Table 3 Growth inhibition of *L. monocytogenes* by competitive exclusion microorganisms in compost extracts at 35°C and room temperature after 24 h and 168 h

Compost	Conc.[a]	Temp	Log difference between LM control and LM+CE during incubation (log CFU/ml)[b]	
			24 h	168 h
Dairy	1:5	RT	1.6	1.9
		35°C	2.2	0.5
	1:10	RT	1.5	0.7
		35°C	1.7	0.1
Poultry	1:5	RT	0.2	0.9
		35°C	0.7	1.1
	1:10	RT	0.3	0.7
		35°C	0.5	1.0

[a] Compost was extracted with water at a ratio of 1 to 5 or 1 to 10.
[b] A cocktail of 3 *L. monocytogenes* strains was inoculated into the compost extract at an initial concentration of ca. 3 log CFU/ml, whereas CE was inoculated at ca. 7 log CFU/ml.

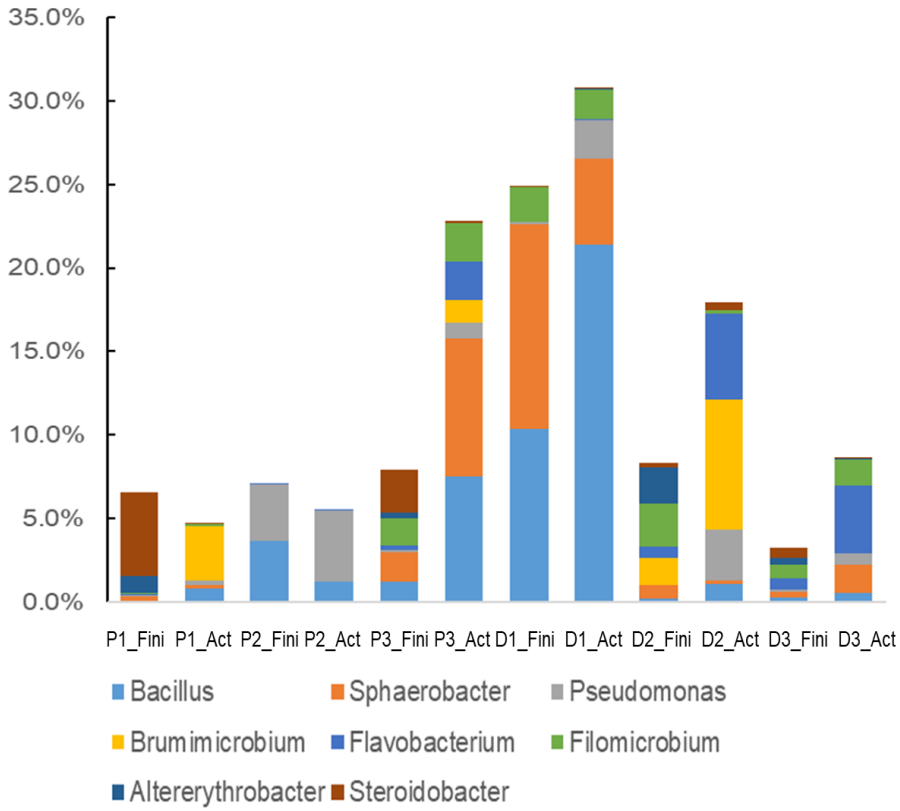

Figure 1 The relative abundance of potential CE microorganisms in compost samples collected from different farms.

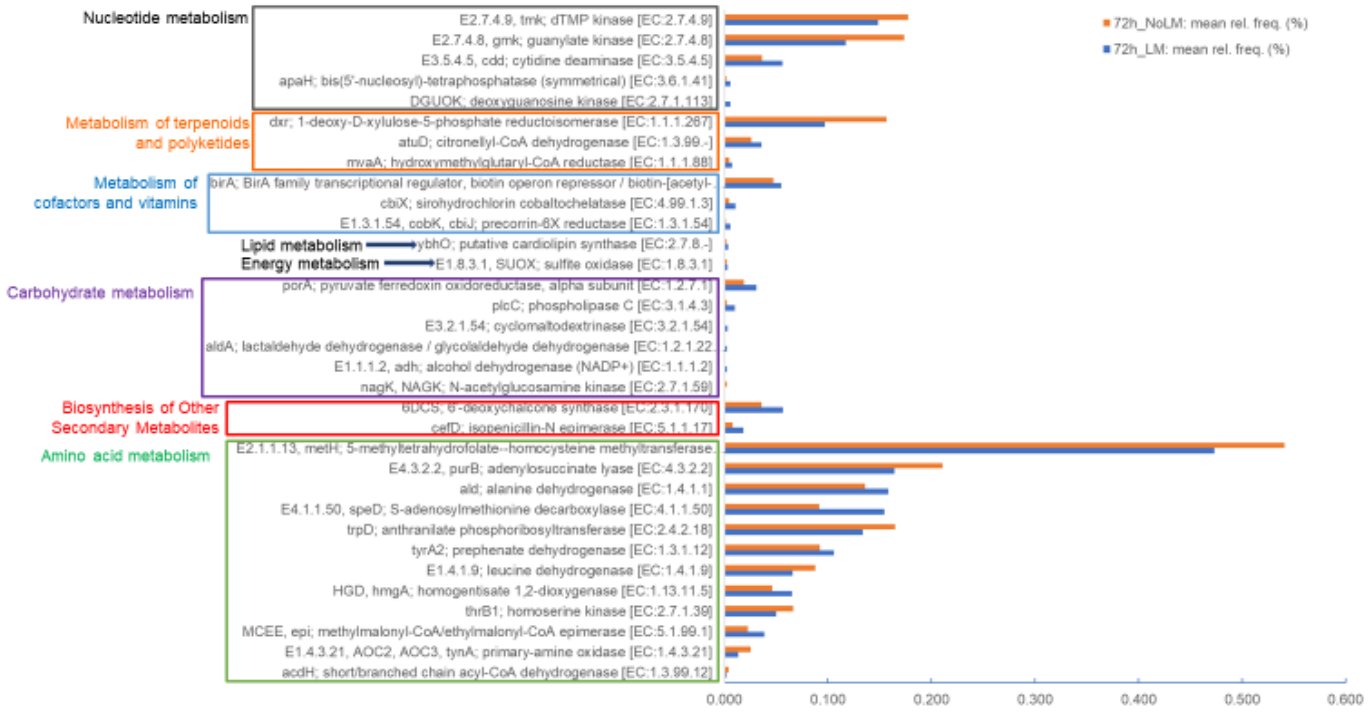

Figure 2 Changes in gene expression associated with selected microbial metabolisms in compost with and without the inoculation of *L. monocytogenes*.

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Abstracts – Presentations and Publication (in preparation)**:**

Hongye Wang, Vijay Shankar, Muthu Dharmasena, and Xiuping Jiang. 2019. Analyzing Microbial Community Change of Turkey Litter Compost Due to Heat Exposure Using 16S High Throughput Sequencing. IAFP Annual Meeting, July 21–24, Louisville, KY

Title: Analyzing microbial community change of turkey litter compost due to heat exposure using 16S high throughput sequencing
Authors: Hongye Wang, Vijay Shankar, Muthu P. Dharmasena, and Xiuping Jiang
Introduction: Turkey litter compost is commonly used as a biological soil amendment. However, there is a lack of knowledge on microbial community changes after heat processing of compost.
Purpose: The objective of this study was to evaluate the microbial profiling in the turkey litter compost before and after heat treatment using 16S high throughput sequencing, and the phylogenetic diversity of the microbial community categorized by analyzing operational taxonomic units (OTUs).
Methods: Turkey litter compost samples before and after dryer were sourced from a commercial plant. Before genomic DNA extraction, DNAs from the dead cells were removed by the propidium monoazide (PMA). The purified DNA samples were sequenced, and high-quality sequenced reads were analyzed using a custom modified QIIME analysis pipeline. Alpha diversity including Shanno, Simpson index, and species richness estimator of Chao I was calculated. Weighted UniFrac was calculated using QIIME, which was used to measure phylogenic beta diversity.
Results: The bacterial community structure in the compost following thermal process was reviewed by the PCoA analysis and UPGMA clustering. The principal co-ordinates 1, 2, and 3, respectively explained 97.41%, 1.79%, and 0.45% of the total structure variations. The clustering analysis showed that the turkey litter compost before the heat treatment was well-clustered together and separated from the heat-treated compost. Taxonomically, the phyla *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most abundant in both before and after heat treatment, whereas *Firmicutes* and *Tenericutes* were predominant in the compost before and after heat treatment, respectively.
Significance: The microbial community analysis method was optimized for turkey litter compost, and the effectiveness of PMA treatment for compost on removing DNA from dead cells was confirmed. Further, the identification of indigenous bacteria in poultry litter surviving the physical heat treatment may lead to future studies on biological control of pathogens in soil amendment.

Hongye Wang and Xiuping Jiang. 2020. Application of Competitive Exclusion Microorganisms to Inhibit the Growth of *Listeria Monocytogenes* in Compost Extract. IAFP Annual Meeting, August 2–5, Cleveland, OH.

Title: Application of competitive exclusion microorganisms to inhibit the growth of *Listeria monocytogenes* in compost extract
Authors: Hongye Wang and Xiuping Jiang
Introduction: Dairy and poultry composts contain a diversity of microbial species. Thus, the compost products can be a good source for isolating compost-borne competitive exclusion (CE) microorganisms which inhibit the growth of pathogens such as *Listeria monocytogenes* (LM).
Purpose: To use various culturing methods to isolate CE microorganisms and then verify antagonistic activities of those CE cultures against LM in compost extract models.
Methods: CE strains were isolated from poultry and dairy composts using triple-agar-layer method, purified, and then identified to species level. The composts were extracted with sterile tap water with ratios of 1:5 and 1:10 (w/v). The filter-sterilized compost extracts inoculated with a cocktail of three LM strains (ca. 3 log CFU/g) were inoculated with or without CE (ca. 7 log

CFU/g), and incubated at room temperature and 35°C up to 168 h. At selected intervals, the samples were enumerated for LM and CE population, respectively.
Results: A total of 40 CE strains were isolated and used in this study. During growth inhibition study, no change ($P > 0.05$) was observed for CE population in poultry compost extract (PCE), but CE population increased 0.27 – 0.56 logs in the dairy compost extract (DCE). After 168 h incubation, the reductions of LM in PCE with extraction ratios of 1:5 and 1:10 were 1.09 and 1.01 logs or 0.85 and 0.73 logs at 35°C or room temperature, respectively. In contrast, the reductions of LM in DCE with extraction ratios of 1:5 and 1:10 were 2.17 and 1.68 logs or 1.61 and 1.79 logs at 35°C or room temperature, respectively. Clearly, the inhibition effect from CE strains increased in more concentrated dairy compost extract (1:5) at 35°C.
Significance: The compost-borne CE microorganisms with anti-*Listeria* activity could control L. monocytogenes contamination in biological soil amendments to ensure safe production of fresh produce.

Hongye Wang, Vijay Shankar, and Xiuping Jiang. Analyze microbial community structure of dairy and poultry compost using phylogenetic marker analysis based on the sequencing of 16S rRNA for bacteria in the presence and absence of *Listeria monocytogenes.* (**in preparation**)

Abstract:
Dairy and poultry composts are important reservoirs for *Listeria monocytogenes*, to reduce *Listeria* contamination, it is essential to understand the ecology of this pathogen where it inhibits. In considering of compost as a rich source of microorganisms with a diversity of microbial species, the interactions between compost native microorganisms and *L. monocytogenes* were studied. A total of 12 biological soil amendments (6 dairy- and 6 poultry-wastes based composts) were collected freshly from 6 compost facilities. The thoroughly mixed compost samples were adjusted to moisture contents as 40 and 80%, and then artificially inoculated with *L. monocytogenes* strain FSL R9-5506 with the optimized inoculation conditions (ca. 7 log CFU/g). At 0 and 72 h incubation period, *L. monocytogenes* population in compost samples was enumerated, and the genomic DNA was extracted, purified, and then subject to phylogenetic marker analysis based on the sequencing of 16S rRNA for bacteria. The comparison of the survival of *L. monocytogenes* in some compost samples among different treatments indicated that regrowth of *L. monocytogenes* occurred in compost samples after 72 h incubation. The major bacterial phyla observed in all farms are *Firmicutes* (23%), *Proteobacteria* (23%), *Actinobacteria* (19%), *Chloroflexi* (13%), *Bacteroidetes* (12%), *Gemmatimonadetes* (2%) and *Acidobacteria* (2%), accounting for 94% of all operational taxonomic units (OTUs) of all compost samples. And *Bacillus*, *Sphaerobactor*, and *Steroidobacter* were found increased in relative abundance after inoculation of *L. monocytogenes* in the compost samples collected from multiple farms.