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Reused poultry litter microbiome with competitive exclusion potential against *Salmonella* Heidelberg

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Abstract

The success of poultry litter reuse in U.S. poultry production can be attributed to the efficient treatment methods used by producers during downtimes (the time lapse between consecutive flocks, during which the broiler house is empty). During this period, reused litter may be decaked, tilled/windrowed, or treated with acid-based amendments to reduce ammonia and bacteria levels. Competitive exclusion, pH, and temperature are proposed factors that influence the level of pathogens and the overall litter microbiome during downtimes. We previously reported on the bacterial genetic factors associated with the fitness of two strains of *Salmonella enterica* serovar Heidelberg (SH) incubated for 14 d in reused litter. Here, we investigated the physicochemical parameters and the microbiome of the litter correlating with SH abundance during this period. We used 16S ribosomal RNA gene sequencing to determine the litter microbiome and whole genome sequencing to characterize bacteria with competitive exclusion potential against SH. The β diversity of the litter microbiome was significantly affected by the duration of incubation, microcosm, and microcosm plus Heidelberg strain combinations. In addition, β diversity was significantly affected by litter parameters, including NH_4, pH, moisture, water activity, and aluminum. The major phyla observed in the reused litter throughout the 14-d incubation experiment were *Firmicutes* and *Actinobacteria*, although their abundance differed by microcosm and time. Amplicon-specific variants homologous to the members of the genera *Nocardiopsis* and *Lentibacillus* and the family *Bacillaceae_2* were found to significantly correlate with the abundance of *Salmonella*. A consortium of *Bacillus subtilis* strains isolated from the litter microcosms reduced the growth of SH in vitro.

Abbreviations: ASV, amplicon sequence variant; BHI, brain heart infusion; CFU, colony-forming units; PBS, phosphate-buffered saline; PLE, poultry litter extract; rRNA, ribosomal RNA; SH, *Salmonella enterica* serovar Heidelberg; WGS, whole genome sequencing.

1 | INTRODUCTION

The United States supports a multi-billion dollar broiler chicken industry and is the world's largest broiler chicken producer (National Chicken Council, 2019). Raising billions of broilers per year produces over 10 million t of litter annually (Cressman et al., 2010). Poultry litter is composed of wood shavings, rice hulls, or sawdust mixed with chicken feces, uric acid, feed, and other broiler-sourced materials. Because of the increasing cost of bedding materials and to reduce the amount of waste produced by broiler houses, producers have opted to recycled bedding materials over multiple flocks for a year or longer (Roll, Dai Pra, & Roll, 2011; Voss-Rech et al., 2017).

The time lapse between consecutive flocks, during which the broiler house is empty (downtime), has been critical to the practice of litter reuse in the United States. During downtimes, reused litter is commonly decaked, tilled/windrowed, or treated with acid-based amendments before use for the incoming flock (Ritz, Fairchild, & Lacy, 2005). These downtime treatments have shown great promise as a cost-effective and viable way to reduce ammonia (NH_3) emissions and to manage built-up litter in broiler grow-out houses (Ritz et al., 2005; Ritz, Fairchild, & Lacy, 2004). For the broiler industry, this has translated to better live production performance, better animal health and welfare, and reduced environmental impact (Li, Lin, Collier, Brown, & White-Hansen, 2013). However, the effect of downtimes on bacterial reduction including *Salmonella* have not been conclusive. The majority of published studies have shown an increase in *Salmonella* or *Escherichia coli* populations after a brief reduction period (~2 wk) (Hunolt et al., 2015; Kassem, Sanad, Stonerock, & Rajashekara, 2012; Wilkinson, Tee, Tomkins, Hepworth, & Premier, 2011; Williams, Blake, & Macklin, 2012).

In our previous study, we showed that *Salmonella enterica* serovar Heidelberg (SH) could persist in reused litter (Oladeinde et al., 2018) and demonstrated that mutations and acquisition of ColE1-like plasmids increased the fitness of SH. The aim of this study was to investigate the litter microbiome and physicochemical properties associated with SH persistence in reused litter.

2 | MATERIALS AND METHODS

Unless otherwise noted, the methods described here have been previously reported in Oladeinde et al. (2018). Poultry litter for microcosm studies was provided by the University of Georgia Poultry Research Center in Athens, GA. The reused litter composed of pine shavings and was previously used to raise three flocks of broiler chickens under simulated commercial poultry production conditions. The litter was collected for microcosm experiments more than 2 wk after bird removal.

Core Ideas

- The microbiome in reused litter microcosms was predictable.
- Physicochemical properties of reused litter significantly affected the microbiome present.
- *Nocardiopsaceae* and *Bacillaceae* were the major bacterial families in reused litter.
- *Bacillus subtilis* inhibited the growth of *Salmonella enterica* serovar Heidelberg.

2.1 | Microcosm setup

Thirty grams of reused litter were placed into 50 250-ml Nalgene wide jars (12 jars per treatment group plus two uninoculated controls), and 1 × phosphate-buffered saline (PBS) (3–4 ml) was added to each microcosm (litter moisture content 32.4 ± 1.6%). Microcosms were incubated for 72 h at 37 °C to allow the establishment and stabilization of the litter microbiome at a comparable level across all treatments. Each microcosm was individually inoculated with approximately 10^6 colony-forming units (CFU) of either Heidelberg strain (SH-2813 or SH-116) preconditioned previously in poultry litter extract (PLE) or brain heart infusion (BHI) broth (serial passing of SH in filter sterilized PLE or BHI; see Supplemental Material) followed by careful mixing. This established a litter moisture content of 27 ± 0.1%, equaling 0.90 ± 0.02 water activity.

2.2 | Bacteriological analyses

After inoculation, three microcosms per treatment group were destructively sampled at Days 0 (immediately after inoculation), 1, 7, and 14. Two uninoculated control microcosms were only sampled at Day 0 due to limited resources. Litter microcosm samples (25 g) were mixed with 150 ml 1× PBS and processed for culturable *Salmonella* as previously described (Oladeinde et al., 2018). Briefly, litter eluate was serially diluted in 1× PBS and plated on XLT-4 and Brilliant Green Sulfur agar. Agar plates were incubated at 37 °C for 24–48 h before CFU was enumerated. Reported counts from XLT-4 agar were log-normalized and reported per gram litter dry weight. The percentage of inoculated *Salmonella* that could be recovered using this method was experimentally derived to be 38 ± 17% (data not shown).

For microbiome determination, 250 mg of litter from each microcosm was extracted in triplicate using a modified Qiagen DNeasy Power-Soil DNA isolation protocol. After the elution step of the protocol, an additional cleanup

with Zymogen PCR inhibitor removal kit (Zymo Research Corp.) was used as per the manufacturer's instructions. All three purified DNA extracts from each microcosm were pooled and concentrated in an Eppendorf Vacufuge Plus Concentrator (Thermo Fisher Scientific) at 30 °C. Pelleted DNA was reconstituted in 100 µl 1× Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5), and concentration was determined fluorometrically using Qubit Fluorometer (Thermo Fisher Scientific). Pooled DNA was used for preparing 16S ribosomal RNA (rRNA) gene libraries for litter microbiome determination.

2.3 | Physicochemical analyses

Litter moisture was determined gravimetrically by drying approximately 1 g of litter at 107 °C for 24 h, and water activity was measured with a WP4C soil water potential meter (METER Group, Inc.). The pH of the litter eluate was measured with a portable Orion Star A series pH meter (Thermo Scientific). The methods used for the measurement of nutrients (total N, total C, total organic C, inorganic C, NH_4, and NO_3) and cations (Fe, Zn, Ca, Cd, Mg, Mn, Cu, Al, Si, P, and Pb) have been previously described (Oladeinde et al., 2018) and are available in the Supplemental Material.

Principal component analysis was performed on all samples using culturable *Salmonella* concentration and litter physicochemical parameters as features. The samples were then projected onto the first two principal components and colored by time, microcosm, and strain metadata to visualize each metadata factor's contribution to the data variance. Principal component analysis was performed using R (R Development Core Team, 2012).

2.4 | Ribosomal RNA gene sequencing

Amplicon sequencing library for all samples was generated as previously described (Allen et al., 2016). Briefly, the V4 hypervariable region of the bacterial 16S rRNA gene was PCR amplified and sequenced using the paired-end (250 × 2) method on the MiSeq platform (Illumina).

2.5 | Microbial community analyses

Filtering, dereplication, sample inference, chimera identification, and paired end read merging of 16S rRNA gene libraries were completed with the Divisive Amplicon Denoising Algorithm (DADA2) R package (Callahan et al., 2016). The product of DADA2 is an amplicon sequence variant (ASV) table that is a high-resolution version of operational taxonomic unit tables produced by popular 16S rRNA gene sequence processing programs like mothur and QIIME. The DADA2 pipeline results in higher-resolution variation between sequences, which improves diversity and dissimilarity analyses downstream (Callahan et al., 2016; Callahan, McMurdie, & Holmes, 2017). Reads were filtered and trimmed using default parameters. The option to pool samples was used to detect rare variants. The naive Bayesian classifier method was used to assign taxonomy to ASVs with the Ribosomal Dataset Project dataset (Cole et al., 2014) with an option to assign species-level information where ASVs matched Ribosomal Dataset Project database sequences exactly. Chimeric sequences were identified and removed using the consensus method with the removeBimeraDenovo command.

The ASV data were imported into the phyloseq package in R (McMurdie & Holmes, 2013). After initial quality filtering, samples with fewer than 5000 reads and ASVs with fewer than five reads were removed. Additionally, taxa identified as Chloroplast or Mitochondria were removed. Observed, Shannon, Chao1, and InvSimpson α diversity measures were calculated and compared using pairwise Wilcoxon tests for nonparametric data and *t* tests for normally distributed data. Nonmetric multidimensional scaling analysis (NMDS) was performed by calculating Bray-Curtis dissimilarities and plotted with the Ampvis2 R package (Andersen, Kirkegaard, Karst, & Albertsen, 2018). The differences in β diversity centroids were conducted with ADONIS tests (function "adonis") and pairwise permutation multivariate ANOVA tests (function "pairwise.perm.manova"). The β dispersion was tested with multivariate homogeneity of groups dispersions (function "betadisper") and tested with permutation tests of multivariate homogeneity of groups dispersions (function "permutest") in R.

The effect of physicochemical variables on microbial community β diversity was analyzed using the envfit function in the vegan package in R (Oksanen et al., 2019). To assess differences in specific taxa abundance, the DESeq2 package in R was used (Love, Huber, & Anders, 2014). DESeq differential abundance analysis uses a negative binomial generalized linear model fitting and Walk statistics for abundance data. Pearson correlation coefficients were calculated in R to examine the correlation between sequence counts of genera *Salmonella* and *Nocardiopsis*, *Lentibacillus*, *Virgibacillus*, *Ruania*, and *Bacillaceae_2*. Counts were $\log_{10}$ transformed, and samples with zero sequences were excluded.

Nocardiopsis ASVs and closely related sequences were aligned using the SILVA Incremental Aligner (SINA) v1.2.11 (Pruesse, Peplies, & Glockner, 2012) before phylogenetic tree construction. The tree was created using the maximum likelihood method implemented in RAxML-NG v. 0.6.0 (Kozlov, Darriba, Flouri, Morel, & Stamatakis, 2019) with the number of bootstrap replicates criteria set to 100. The best model of sequence evolution predicted by jModelTest

(Darriba, Taboada, Doallo, & Posada, 2012) was used for tree reconstruction.

2.6 | Isolation and identification of "filamentous-like" bacteria from reused litter

During the course of the 14-d experiment, we observed a bloom of "filamentous-like" colonies on Brilliant Green Sulfur agar starting at Day 1 (Supplemental Figure S1). This made us question whether this bloom was associated with SH inoculation. Based on this premise, we retrospectively screened saved litter slurry from this study (stored in RNAlater [Thermo Fisher Scientific] at −80 °C) for bacteria with "filamentous-like" morphology. To do this, a 10-µl inoculating loop was used to streak litter slurry onto BHI agar. Afterward, five pure colonies recovered from five different SH-2813 + BHI microcosms were randomly selected for whole genome sequencing (WGS). Genomic DNA was extracted from pure cultures using a Qiagen DNeasy Power-Soil DNA isolation kit and used for WGS library preparation.

The WGS libraries were prepared using a MiSeq Nextera XT library preparation kit. Sequencing was performed on the Illumina MiSeq platform with 150-bp paired end reads using the MiSeq reagent V2 (300 cycles). All isolates were sequenced for an average coverage >100×. Sequence reads were assembled de novo into contigs using Unicycler v.0.4.7 (Wick, Judd, Gorrie, & Holt, 2017). Assembled contigs were submitted to the Center for Genomic Epidemiology's CCMetagen (Marcelino et al., 2019) for bacterial identification. In addition, sequenced genomes were classified using the GTDB-Tk software based on the genome taxonomy database GTDB with default parameters (Chaumeil, Mussig, Hugenholtz, & Parks, 2019; Parks et al., 2018). Assembled contigs were also checked for similarity to known reference genomes using Mash v2.2 and the RefSeq sketch database provided on the Mash documentation website (k21s1000) (Ondov et al., 2019). Complete 16S rRNA sequences from the five genomes were blasted against the nonredundant National Center for Biotechnology Information (NCBI) database, and the 16S rRNA gene sequences of the 100 closest neighbors were extracted.

2.7 | Comparative analysis of whole genomes

PlasmidFinder v. 2.0 (Carattoli et al., 2014) and Unicycler were used to determine the plasmid replicon types present. The Comprehensive Antibiotic Resistance Database (Jia et al., 2017) was used to identify antibiotic resistance genes, and PHAST (Zhou, Liang, Lynch, Dennis, & Wishart, 2011) was used for bacteriophage identification. Assemblies were annotated with Prokka (Seemann, 2014) using the default settings and by performing a PSI-BLAST search against the nonredundant NCBI database. KofamKOALA (Aramaki et al., 2019) was used to identify complete KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (molecular interaction maps) that the sequenced genomes are capable of. A pan genome analysis of annotated assemblies and closely related genomes identified by CCMetagen, GTDB-Tk, and Mash was conducted with Roary (Page et al., 2015). A phylogenetic tree based on the core genome, the accessory genome, and 16S rRNA sequences was reconstructed using the maximum likelihood method implemented in RAxML-NG.

2.8 | Growth inhibition assay

After identifying the "filamentous-like" bacterial strains as *Bacillus subtilis*, we questioned if they were capable of inhibiting the growth potential of SH-2813 (see Supplemental Material for detailed description of the assay). Briefly, one bacterial colony per *Bacillus* strain ($n = 5$) was resuspended in 900 µl of 1× PBS. Thereafter, 10 µl of each suspension ($\sim 10^6$ CFU) was transferred into 900 µl of 1:100 PLE (pH 9.0) and incubated at 37 °C for 2 h. After incubation, 50 µl of SH-2813 ($\sim 10^4$ CFU) was inoculated into each sample containing the mixture of *Bacillus* strains and controls with no *Bacillus*. This resulted in a 1:100 ratio of SH-2813 to *B. subtilis* population. Afterward, all samples were incubated for an additional 24 h. The concentration of SH-2813 was determined at 0, 1, and 24 h after inoculation ($n = 3$ per time point) by direct plating of serial dilutions onto xylose lysine deoxycholate agar. The concentration of *Bacillus* was estimated by direct plating onto BHI plates.

3 | RESULTS

3.1 | Physicochemical properties of litter microcosms

After the 72-h incubation of litter microcosms and immediately after *Salmonella* inoculation (Day 0), the moisture (28.2 ± 0.04%), pH (8.88 ± 0.03), water activity (0.917 ± 0.01), and NH_4 (18,683 ± 1409 mg kg^{-1}) were higher in microcosms inoculated with BHI-preconditioned SH strains than PLE-preconditioned strains (24.9 ± 0.04, 8.08 ± 0.14, 0.898 ± 0.04, 8380 ± 909) (Supplemental Table S1). This difference in litter properties at Day 0 also correlated with the location of the microcosms in the incubators used. Microcosms positioned in the upper shelf of the incubator (BHI) had significantly higher physicochemical parameters than microcosms positioned in the lower shelf of incubator

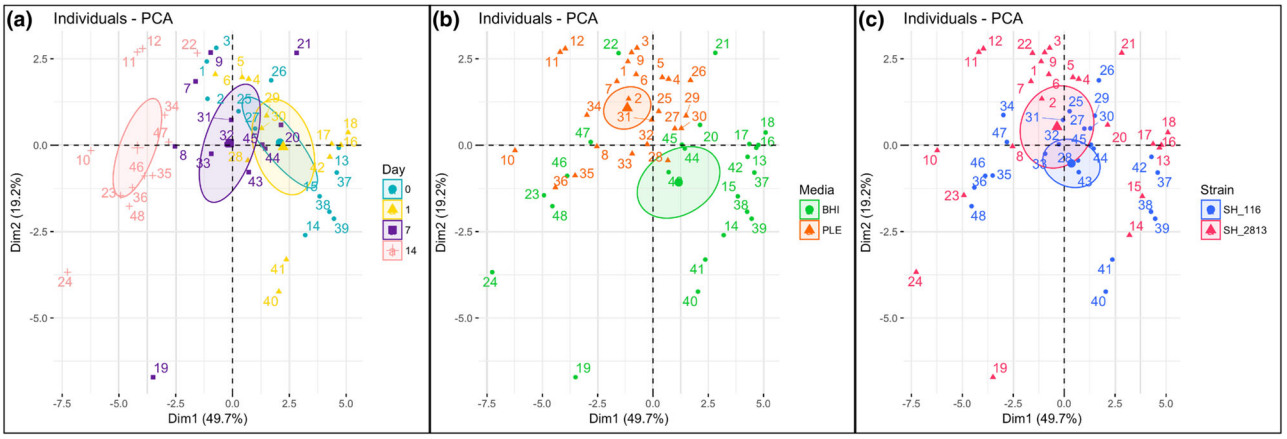

FIGURE 1 Principal component analysis (PCA) characterizing the differences between microcosms grouped by (a) days, (b) treatment, (c) and *Salmonella* Heidelberg strains. The PCA was constructed using all physicochemical parameters measured and *Salmonella* concentrations. Each number and dot denote one microcosm, and data represents 48 microcosms. The farther apart two samples are from each other, the more different they are. Ellipses represents the mean/confidence interval if all the dots of the same color are represented by just one sample.

(PLE); this is referred to as the "incubator effect." Therefore, it is difficult to ascertain if preconditioning of strains was responsible for such a significant difference at Day 0. Nevertheless, we will continue to refer to these microcosms as PLE-preconditioned strains/microcosms and BHI-preconditioned strains/microcosms.

A principal component analysis revealed that microcosms differed by days of incubation (Figure 1a) and by preconditioned strains/microcosms (BHI vs PLE) (Figure 1b) but not by strain only (Figure 1c). Furthermore, the litter physicochemical variables could explain up to ~70% (Dim 1 and Dim 2) of the variability in our data, suggesting that the remaining variability in our data can be attributed to the microbiome and other unmeasured variables.

3.2 | *Salmonella* concentration in reused litter

Based on *Salmonella* direct counts, culturable *Salmonella* concentration declined by several logs 1 d after inoculation into reused litter. This decline was significantly ($p = .004$; Wilcoxon signed-rank test) higher (up to 6 logs) for BHI-preconditioned strains/microcosms compared with PLE-preconditioned strains/microcosms (~3 logs) (Table 1). At Day 7, an increase in *Salmonella* population was observed for BHI-preconditioned strains/microcosms before a subsequent decrease at Day 14 (Table 1). Culturable *Salmonella* was higher in microcosms inoculated with SH-2813 compared with SH-116 at Day 14 ($p = .002$; Wilcoxon signed-rank test). *Salmonella* was detected by 16S rRNA sequencing and was present in a higher abundance in SH-2813 PLE-preconditioned strains/microcosms than in SH-2813 BHI-preconditioned strains/microcosms ($p = .032$) (Table 1).

3.3 | Reused litter microbiome diversity

For 16S rRNA gene bioinformatic analyses, an average of 23,551 sequences per sample were retained after initial quality filtering, trimming, de-noising, and chimera checking of 16S rRNA gene sequencing raw reads. The total number of sequences per sample ranged from 6,589 to 39,642 (Supplemental Figure S2). Approximately 600 true sequence variants (unique sequences) were found in each sample's forward and reverse reads after the core sample inference algorithm. A total of 59 chimeric sequences were detected, and 97.73% of data was kept after chimera removal.

The α diversity was consistently lower in BHI-preconditioned strains/microcosms than in PLE-preconditioned strains/microcosms ($p < .01$) (Figure 2). The α diversity was not significantly influenced by the SH strain inoculated; however, SH-2813 + PLE microcosms had significantly higher Shannon ($p = .01$) and InvSimpson ($p = .001$) α diversity measures than SH-2813 + BHI microcosms (Supplemental Figure S3). The SH-2813 + PLE microcosm was also higher than the SH-116 + BHI across all α diversity measures ($p < .01$) (Supplemental Figure S3). The α diversity of the litter microbiome did not change significantly over the course of the experiment.

The β diversity, measured as distance to group centroid, was found to be affected by days and microcosms ($p = .001$) (Figure 3). The β diversity was significantly different between BHI-preconditioned strains/microcosms and PLE-preconditioned strains/microcosms ($p = .003$) (Figure 3a); however, β diversity was not significantly influenced by the Heidelberg strain alone (Figure 3b). In addition, β diversity significantly differed between days ($p < .05$) (Figure 3c) and between microcosm/Heidelberg strain combinations ($p < .05$) (Figure 3d).

TABLE 1 *Salmonella* abundance in poultry litter microcosms as determined by culture and 16S ribosomal RNA (rRNA) gene analyses

Treatments/microcosms[a]	Day	*Salmonella*[b]	Number of raw 16S rRNA gene sequence reads
		Log CFU[c] per g dry weight	
SH-2813 + PLE	0	6.10 (0.02)[d]	41.33 (17.78)
	1	1.91 (0.05)	45 (17.08)
	7	3.43 (0.22)	30.66 (36.90)
	14	2.47 (0.03)	35 (23.64)
SH-116 + PLE	0	5.64 (0.06)	17.33 (12.42)
	1	2.81 (0.18)	35 (9.84)
	7	2.33 (0.50)	25.33 (7.50)
	14	<LOQ = 2.83 (2.13)[e]	26 (5.29)
SH-2813 + BHI	0	5.42 (0.06)	56 (0.69)
	1	<LOQ = 7 (0.04)[e]	2.33 (2.08)
	7	3.45 (0.04)	1.33 (2.30)
	14	2.51 (0.05)	1.33 (1.52)
SH-116 + BHI	0	5.81(0.14)	87.33 (47.43)
	1	<LOQ = 7 (0.09)[e]	48.33 (73.43)
	7	2.29 (0.22)	15.66 (3.78)
	14	<LOQ = 2.88 (2.02)[e]	13.33 (7.23)
Uninoculated control	0	<LOD	0 (0)

[a]BHI, brain heart infusion; PLE, poultry litter extract. [b]*Salmonella* concentration was adopted from Figure 1 of Oladeinde et al. (2018). Limit of quantification (LOQ) and limit of detection (LOD) for culturing of *Salmonella* from litter were ∼26 and 5 CFU/g dry weight, respectively (LOQ and LOD were experimentally derived). [c]Colony-forming units. [d]Values are mean (SD). [e]Number reported is concentration after overnight enrichment in buffered peptone water.

The β diversity at Day 0 for BHI-preconditioned strains/microcosms was significantly affected by NH_4, Al, Fe, pH, Zn, and total organic C, whereas the β diversity of PLE-preconditioned strains/microcosms was affected by Mg (Supplemental Figure S4a). Aluminum and *Salmonella* concentrations of the litter microcosms significantly affected the β diversity of PLE-preconditioned strains/microcosms (Supplemental Figure S4b).

3.4 | Dominant phyla present in reused litter

Amplicon sequence variants present in litter microcosms differed by day, Heidelberg strain, and microcosm. Amplicon sequence variants homologous to the members of the phyla *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were present at a relative abundance of ∼64, 33, and 1.5% at Day 0 for all treatments (Figure 4**;** Supplemental Figures S5 and S6). The *Firmicutes* were represented by members of the genera *Thermoactinomyces*, *Enterococcus*, *Oceanobacillus*, *Staphylococcus*, *Gracibacillus*, *Lentibacillus*, *Virgibacillus*, *Sporosarcina*, *Atopostipes*, *Salinicoccus*, *Lactobacillus*, *Aerococcus*, and unidentified members of the family *Bacillaceae_2*. *Actinobacteria* included the genera *Nocardiopsis*, *Brachybacterium*, *Yaniella*, *Corynebacterium*, *Haloactinobacterium*, *Enteractinococcus*, *Brevibacterium*, *Tomitella*, and *Ruania*. *Bacteroidetes* were represented by *Sphingobacterium*, *Balneola*, and an unidentified ASV belonging to the family *Chitinophagaceae. Salmonella* accounted for ∼36% of the relative abundance of ASVs identified for phylum *Proteobacteria* at Day 0. In addition, members of the genera *Halomonas*, *Paenalcaligenes*, and *Lysobacter* represented *Proteobacteria*. Phyla *Deinococcus-Thermus*, *Chloroflexi*, *Euryarchaeota*, *Fusobacteria*, *Spirochaetes*, *Synergistetes*, and *Verrucomicrobia* were present at a relative abundance of <0.1% (Figure 5; Supplemental Figure S7a–d).

Firmicutes population decreased and *Actinobacteria* increased in BHI-preconditioned strains/microcosms from Day 0 to Day 7 (Figure 4). The observed higher abundance of *Actinobacteria* in BHI-preconditioned strains/microcosms was associated with ASVs homologous to *Nocardiopsis, Ruania*, *and Haloactinobacterium. Lentibacillus, Virgibacillus*, and unidentified members of the family *Bacillaceae_2* represented the major *Firmicutes* present in BHI-preconditioned strains/microcosms (Figure 5). In contrast, *Thermoactinomyces*, *Enterococcus*, *Oceanobacillus*, *Staphylococcus*, *Gracilibacillus*, *Nosocomiicoccus*, *Ornithinibacillus, Lactobacillus, Salinicoccus, Jeotgalicoccus*, and unidentified members of the family *Bacillaceae_2* represented *Firmicutes* of higher abundance in PLE-preconditioned strains/microcosms. *Brevibacterium*, *Yaniella*, *Brachybacterium*, *Corynebacterium*, and *Enteractinococcus* were

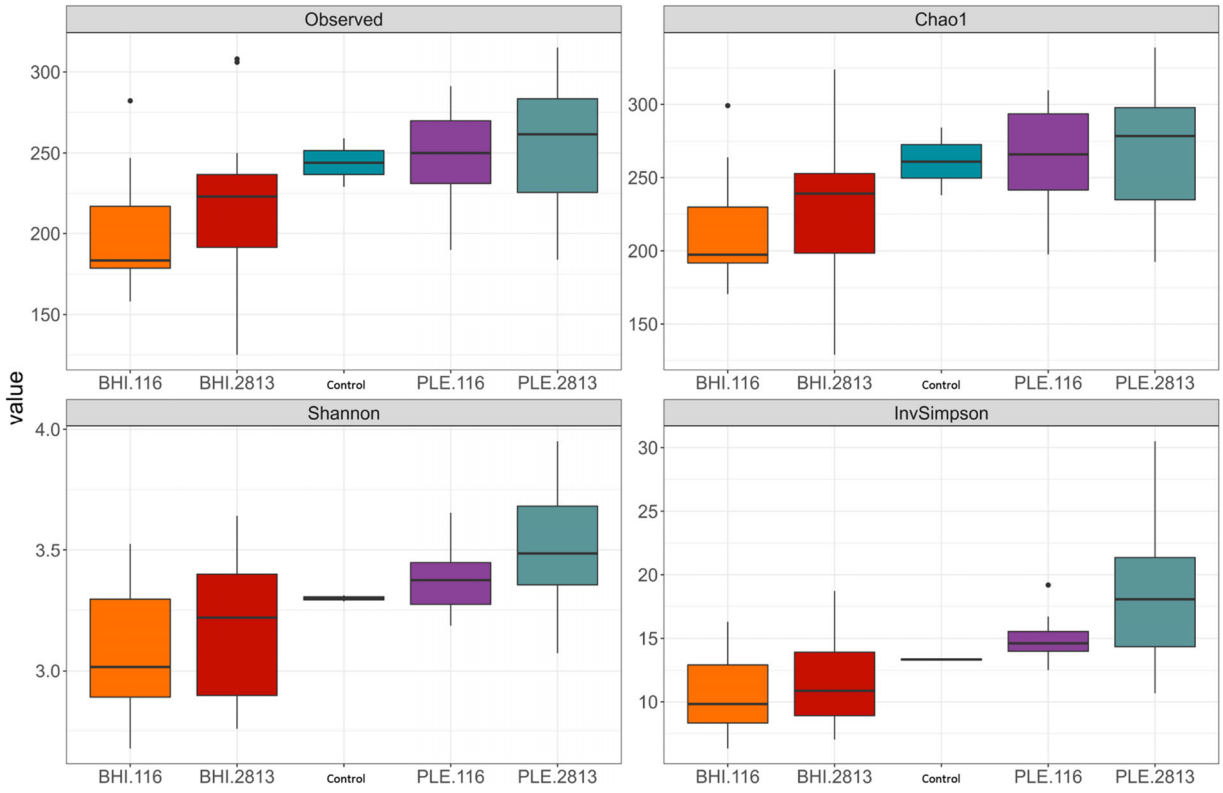

FIGURE 2 Microbial α diversity indices determined based on 16S ribosomal RNA gene libraries for samples collected from microcosms with different treatments. Boxes indicate the interquartile range (75th to 25th) of the data. The median value is shown as a line within the box. Whiskers extend to the most extreme value within 1.5 times the interquartile range. BHI, brain heart infusion; PLE, poultry litter extract.

the *Actinobacteria* genera of higher abundance in PLE-preconditioned strains/microcosms.

3.5 | Reused litter microbiome correlating with *Salmonella* Heidelberg abundance

No *Salmonella* was detected in uninoculated control samples by 16S rRNA gene sequencing or by culture method, suggesting that the *Salmonella* ASVs identified belong to the inoculated SH strains. *Salmonella* was higher in PLE-preconditioned strains/microcosms compared with BHI-preconditioned strains/microcosms from Day 1 to Day 14. In addition, *Salmonella* in BHI-preconditioned strains/microcosms experienced a significant decline (below the limit of quantification) in culturable populations compared with PLE-preconditioned strains/microcosms after 1 d of incubation (Table 1). At Day 1, the ASVs in BHI-preconditioned strains/microcosms differed significantly from PLE-preconditioned strains/microcosms. For instance, only members of three genera (*Nocardiopsis*, *Virgibacillus*, and *Lentibacillus*), one class (*Betaproteobacteria*), and family *Bacillaceae_2* had significantly higher abundance in BHI compared with PLE. In contrast, PLE-conditioned strains/microcosms had members of 14 genera and one family in higher abundance compared with BHI microcosms (Figure 5; Supplemental Figure S7a–d).

A Pearson correlation test between *Salmonella* and the most abundant ASVs in BHI microcosms at Day 1 revealed that *Nocardiopsis* ASVs were negatively correlated with *Salmonella* abundance ($r = -.43$; $p = .059$), whereas *Lentibacillus*, *Virgibacillus*, and *Bacillaceae_2* were positively correlated ($p < 0.1$) (Table 2). Based on the observed negative correlation for *Nocardiopsis*, we sought to further determine the taxonomy of ASVs associated with *Nocardiopsis*. A phylogenetic tree reconstructed (Supplemental Figure S8) using the SILVA Alignment, Classification, and Tree Service (SILVA ACT) revealed that the ASVs are novel and diverse (clade highlighted in green) and are closely related to *N. alkaliphila, N. kunsanensis*, and *N. litoralis* (taxa colored in red).

3.6 | Genotypic features of "filamentous-like" *Bacillus subtilis*

Phylogenetic trees reconstructed using whole genome (core and accessory genes) and 16S rRNA gene sequences suggest that the closest relative to the "filamentous-like" bacteria isolated from the litter microcosms was *B. subtilis* (Figure 6; Supplemental Figure S9). The closest subspecies identified

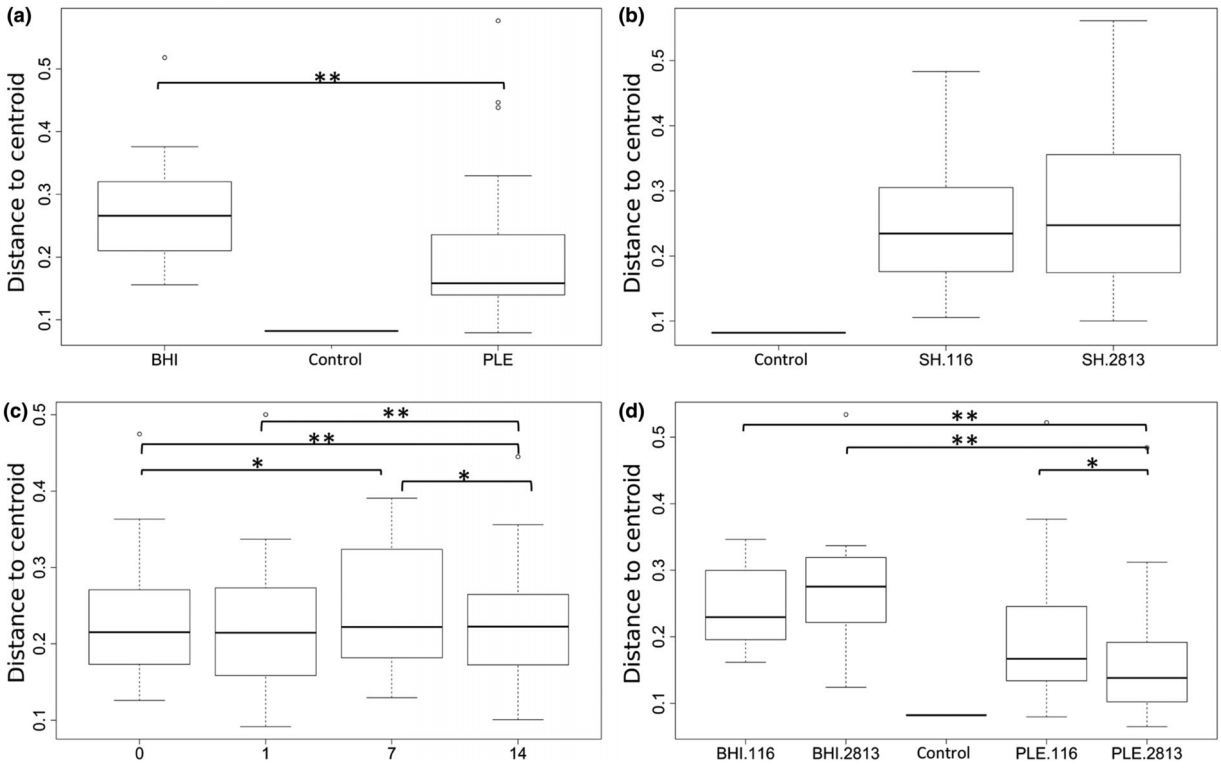

FIGURE 3 Variability in bacterial community structure (assessed by analysis of β dispersion, a metric of variability) between treatments. Comparisons of β diversity between (a) brain heart infusion (BHI) and poultry litter extract (PLE) microcosms, (b) Heidelberg strains, (c) days, and (d) microcosm/Heidelberg strain combinations. On the boxplots, the center lines show the medians, the bottom and upper limits indicate the 25th and 75th percentiles, and whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Levels of significance: ns: $p > .05$, *$p \leq .05$, **$p \leq .01$.

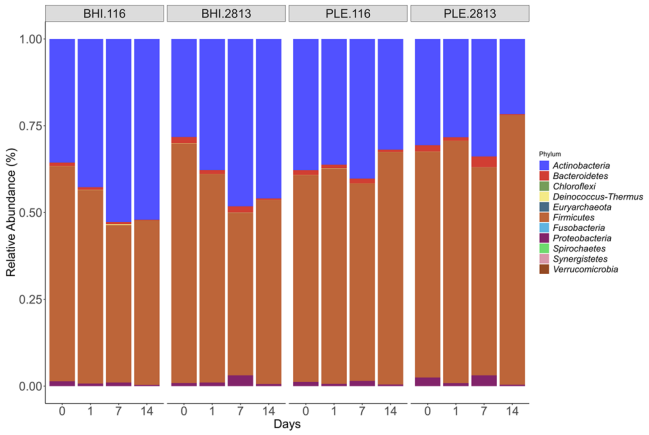

FIGURE 4 Phylum-level classification of 16S rRNA gene sequence reads based on treatment and day of sampling. Data represent average of amplicon sequence variant (ASV) counts from replicate libraries for each category. BHI, brain heart infusion; PLE, poultry litter extract.

based on 16S rRNA gene sequence alone were subspecies *Spizizenii* and *Inaquosorum*. Their genomes were on average 4,012,662 base pairs long, and ~4,020 genes were predicted as the protein coding DNA sequence. The strains carried no plasmid replicons, but three bacteriophages were identified. Numerous types of antimicrobial resistance genes, including genes known to confer resistance to metals and disinfectants, were predicted (Supplemental Table S2). In addition, the strains harbored complete KEGG pathway genes required for the biosynthesis of bacilysin and kanosamine, two well-studied bioactive secondary metabolites (Supplemental Table S3).

3.7 | Growth inhibition of *Salmonella* Heidelberg by *Bacillus subtilis*

The consortium of five *B. subtilis* strains from this study significantly reduced the growth of SH-2813 by an average 0.2 log units (~10^7 CFU ml^{-1} difference) ($p = .086$; Wilcoxon signed-rank test) after 24 h of growth in filter-sterilized PLE (pH 9) (Supplemental Figure S10).

4 | DISCUSSION

Microcosms are widely used to study microbial communities in complex systems such as soil and poultry litter. In most cases, microcosms are the only alternatives to in situ

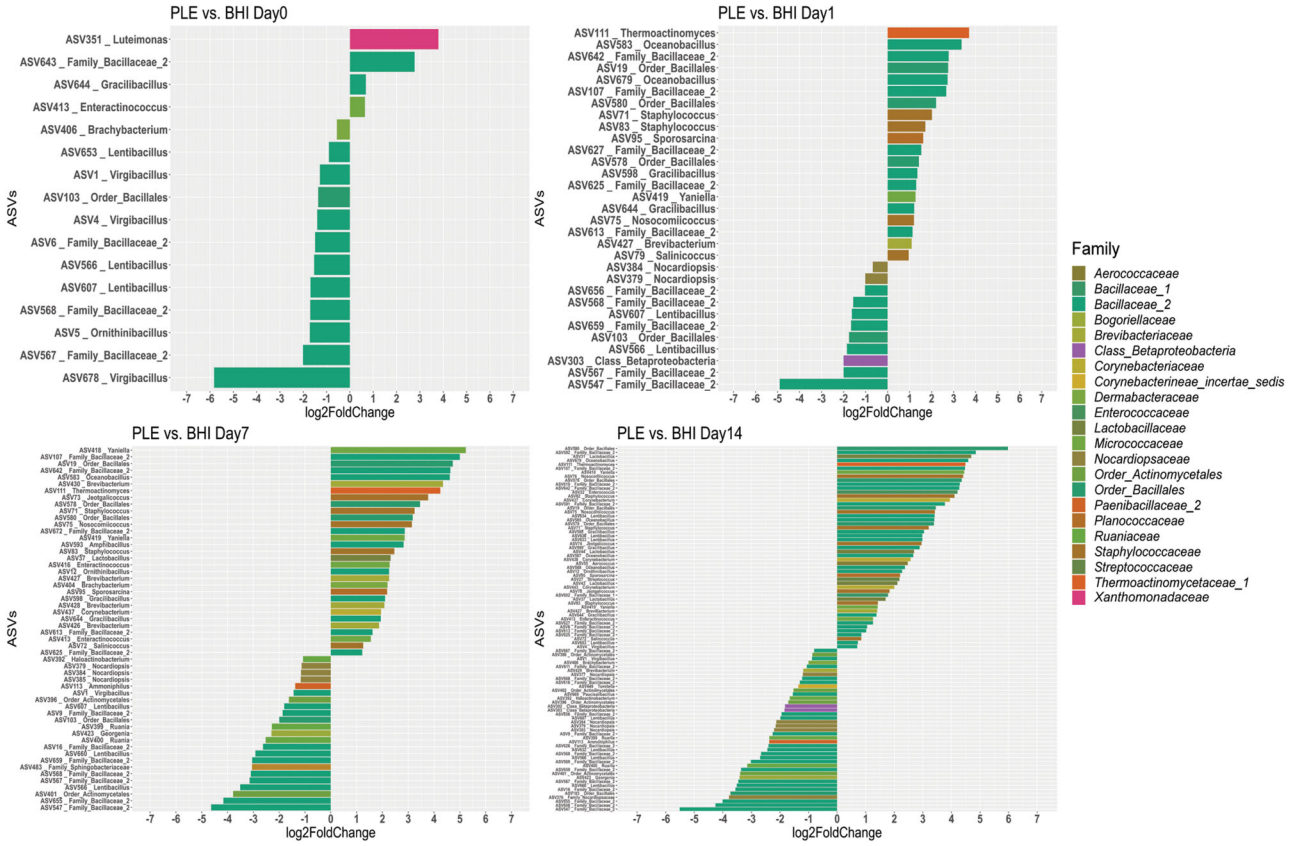

FIGURE 5 Plots of amplicon sequence variants (ASVs) that were significantly differentially abundant ($p_{adj} < .05$) according to DESeq2 analysis. Significant ASVs are plotted individually and colored according to their family-level classification.

TABLE 2 Correlations between *Salmonella* and taxa that have been found to be significantly affected by brain heart infusion (BHI) or poultry litter extract (PLE) treatment

		BHI		PLE	
Group 1	**Group 2**	***R***	***P* value**	***R***	***P* value**
Salmonella	*Nocardiopsis*	−.43	.059	.32	.13
	Bacillaceae_2	−.04	.85	.4	.05
	Lentibacillus	.45	.028	.49	.014
	Virgibacllus	.27	.21	.36	.087
	Ruania	−.23	.27	−.057	.79

controlled experiments where inoculation of broiler houses with pathogens are either prohibitive or nonreproducible. The main argument against the use of microcosms is the risk of investigating artifacts of the system (Juyal et al., 2018). For instance, Juyal et al. (2018) demonstrated that the growth of *Bacillus* and *Pseudomonas* sp. inoculated into soil microcosms differed significantly by the bulk density of the soil. In this study, we observed that the incubator represented an artifact altering SH population dynamics.

One of the objectives of this study was to determine if preconditioning Heidelberg strains in filter-sterilized PLE would confer better survival upon inoculation into reused litter than preconditioning in BHI broth. However, we observed that litter properties differed significantly at the time of inoculation (Day 0) between microcosms that were incubated for 72 h in the upper shelf of the incubator (BHI) versus the lower shelf (PLE) prior to SH inoculation. It is more likely that the significant differences on Day 0 resulted from what we have termed "the incubator effect" rather than preconditioning of Heidelberg strains in different media. Moreover, a single-nucleotide polymorphism–based tree for 86 SH isolates recovered from this study did not reveal genotypic differences between SH isolates recovered from BHI compared with PLE microcosms on Day 0 ($n = 15$) (Oladeinde et al., 2018). Significant temperature variation has been reported within (front and back) and between incubators (Walker, Butler, Higdon, & Boone, 2013), suggesting that the "incubator effect" should be considered when planning a study involving the use of microcosms.

The incubator effect resulted in two significantly different microcosms at Day 0. As expected, SH strains exhibited different population dynamics upon exposure to poultry litter microcosms. This difference was also evident from the 16S rRNA gene analysis, where BHI-preconditioned strains/microcosms (microcosms on the upper shelf of the incubator) showed less diversity and were more dispersed

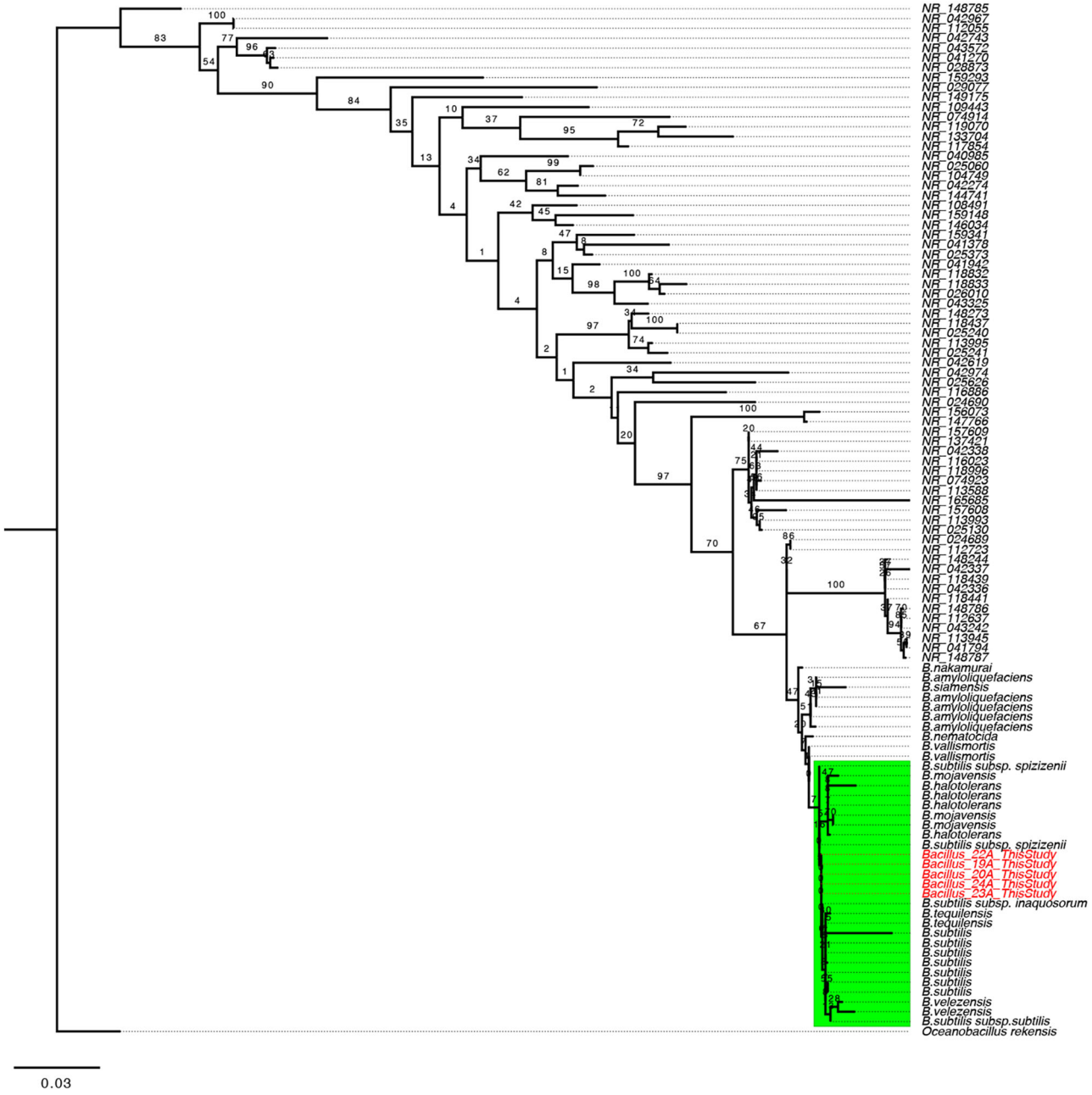

FIGURE 6 Maximum likelihood tree of full-length 16S ribosomal RNA gene sequences of five "filamentous-like" bacteria from this study associated with the genus *Bacillus* and their closest neighbors. The *Bacillus* strains from this study (highlighted in red) formed a distinct clade, with *Bacillus subtilis* indicated by the green box. The GTR+FO+I model of nucleotide substitution and the GAMMA model of rate heterogeneity were used for sequence evolution prediction. Numbers shown next to the branches represent the percentage of replicate trees where associated taxa cluster together based on ~100 bootstrap replicates. Trees were rooted with the most distant relative, *Oceanobacillus rekensis* (NCBI accession number: NR_145558).

and variable than PLE-preconditioned strains/microcosms (microcosms on the lower shelf of the incubator). This alludes to the Anna Karenina hypothesis for animal microbiomes (Zaneveld, McMinds, & Vega Thurber, 2017). Instead of the microbiome following a predictable successional pattern like PLE-preconditioned strains and microcosms, BHI-preconditioned strains and microcosms were stochastically influenced by higher physicochemical parameters when compared with PLE treatments.

One interpretation of the Anne Karenina hypothesis outside the context of animal-associated microbiomes is that environmental stressors disrupt the normal mechanisms that regulate the interactions between bacterial members of a community (Lamont & Hajishengallis, 2015; Zaneveld et al., 2017).

Physiochemical parameters can alter the complex interplay between beneficial species, potential pathogens, and environmental factors, drawing on the ecological theory of community assembly and succession (Byrd & Segre, 2016; Nelson, De Soyza, Perry, Sutcliffe, & Cummings, 2012; Vayssier-Taussat et al., 2014). This may explain why this study observed a seemingly counterintuitive trend that BHI microbiomes achieved a significantly lower abundance of *Salmonella* than PLE microbiomes after 1 d of incubation. Higher physicochemical parameters in BHI treatments that led to the dispersion of BHI microbiomes simultaneously increased ASV abundances of select genera. It is plausible that the "dysbiotic" nature of BHI microbiomes (described as a state of imbalance) decreased the abundance of unknown ASVs and increased the abundance of genera capable of excluding *Salmonella* from litter (Kamada, Chen, Inohara, & Nunez, 2013).

Furthermore, a Pearson correlation test revealed that the *Salmonella* population exhibited a negative correlation with *Nocardiopsis* and a positive correlation with ASVs from family *Bacillaceae*. *Nocardiopsis* sp. has been shown to produce a variety of bioactive compounds, including antibiotics, surfactants, and toxins (reviewed in Bennur, Kumar, Zinjarde, & Javdekar [2015] and Ibrahim et al. [2018]). Members of the genus *Bacillus* have been used extensively as direct-fed microbials for chickens (Grant, Gay, & Lillehoj, 2018). Likewise, we identified *B. subtilis* strains with "filamentous-like" colony morphology. These strains increased in abundance after inoculation of microcosms with SH and inhibited the growth of SH-2813 in vitro.

An effective downtime is critical for the success of the next flock of birds that will be raised on reused litter. Beginning from approximately Day 1, chicks begin pecking at and consuming litter materials, thereby inoculating their young gut with microbiota present in the litter (Wang, Lilburn, & Yu, 2016). Consequently, reused litter available for a subsequent flock after a downtime will have a significant effect on the developmental process of new chicks and their microbiome. Downtime treatments that can reduce pathogen populations will have a greater potential in limiting pathogen transfer to the incoming flock. For the farmer, a shorter downtime will result in growing more birds throughout the year. However, little is known of the microbiome of downtime litter or of their interactions with pathogens such as *Salmonella*.

Davitt (2014) performed the most in-depth reused litter microbiome analysis to date. In this study, reused litter was collected during downtimes and from "long-term stored" litter (stored 6 wk to 3 mo after birds were removed). The litter was collected from 13 broiler houses from three U.S. states and was composed of either sawdust, wood shavings, or rice hull. Despite these geographical and physiological differences, the litter microbiome was not significantly different between producers/integrators or between bedding materials ($p > .05$), suggesting that reused litter harbors a consistent and predictable microbiome. Davitt (2014) showed that *Bacillaceae, Brevibacteriaceae, Dermabacteriaceae*, and *Nocardiopsaceae* were the major families inhabiting reused litter, which supports the results from this study. Our study also shows that reused litter microcosms harbor a consistent and predictable microbiome regardless of initial variability due to the "incubator effect." For example, irrespective of the physicochemical conditions and the presence or absence of particular *Salmonella* strains, BHI and PLE treatments were predictable in the members of the community that persisted by Day 14.

From a farm management perspective, this group of bacteria may serve as a biological indicator of reused litter that has undergone an adequate downtime. For example, high levels of *Nocardiopsis* or family *Bacillaceae* and low levels of *E. coli* indicate that downtime litter can be used for the incoming flock, whereas the opposite is true for a litter that requires a longer downtime. Alternatively, litter bacteria (e.g., *Nocardiopsis* and *Bacillus*) may be useful as poultry "litterbiotics" to facilitate the downtime process for reused litter.

The microbiome of the litter used for this study is not representative of all reused litter considering there are multiple factors associated with litter microbiome succession, particularly the physicochemical variables. The numbers of flocks raised on the litter prior to "build-up" and the length of the downtime are examples of management practices that may influence microbial succession. In addition, the incubator was identified as a possible source of variability in this study. Nonetheless, our study demonstrated how significant changes in nutrients, pH, water activity, and moisture can shift the bacterial diversity of reused litter from a predictable microbiome to a "dysbiotic" system favoring only a few selected genera and families.

ETHICS STATEMENT

The authors did not physically interact with chickens raised on the poultry litter used in this study; therefore, they were exempt from university guidelines regarding Institutional Animal Care and Use Committee – University of Georgia Office of Animal Care and Use and USDA/NIH regulations regarding animal use.

DATA AVAILABILITY

All sequencing data are available under NCBI Sequence Read Archive BioProject: PRJNA448609.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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