Poultry Litter Microbiome Shifts in Commercial Broiler Houses Following a Biotic-Based Litter Treatment

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Abstract

Litter management plays a critical role in broiler production, affecting bird health, performance, and environmental impact. This preliminary study evaluated the effects of IndigoLT™, a biological amendment, in combination with a 40% sodium bisulfate ($NaHSO_4$) regimen on litter microbiome composition, compared to a standard $NaHSO_4$-only treatment. Litter samples ($n = 18$) were collected from three commercial broiler houses following flock removal: one control ($NaHSO_4$-only), one treated with IndigoLT for a single flock, and one treated for two consecutive flocks. The V4 region of the 16S rRNA gene was sequenced to assess prokaryotic community composition. Alpha diversity metrics (Faith's phylogenetic diversity, observed ASVs, Pielou's evenness, Shannon diversity) did not differ significantly across treatments (ANOVA, $p > 0.05$). In contrast, PERMANOVA analyses of Bray-Curtis, Jaccard, Weighted and Unweighted UniFrac distances revealed significant shifts in β diversity between IndigoLT-amended and control groups ($q < 0.05$), with no significant differences between the one- and two-flock IndigoLT treatments. Core microbiome and differential abundance analyses suggested that IndigoLT, when paired with reduced $NaHSO_4$ input, may accelerate organic matter decomposition, promote nitrogen retention, and suppress potentially pathogenic taxa. Although limited by the absence of baseline sampling and biological replication, these findings suggest that IndigoLT influences litter microbial succession. Future work should aim to optimize inclusion rates most complementary between $NaHSO_4$ and IndigoLT to enhance litter quality, reduce NH_3 volatilization, and support bird health.

Keywords: Broiler litter, Microbiome, IndigoLT, 16S rRNA sequencing, Litter amendment

Introduction

Poultry litter management is critical to broiler production systems, directly impacting bird health and welfare, environmental sustainability, and food safety. Litter serves as a biologically complex substrate composed of fecal matter, feed residues, bedding material, and diverse microbial populations that interact dynamically with environmental and management factors. These microbial communities are shaped by variables such as stocking density, litter age, and the application of chemical or biological amendments (Bolan et al., 2010). As the poultry industry shifts toward antimicrobial stewardship and microbiome-informed production strategies, characterizing the microbial ecology of broiler litter has gained prominence (Pedroso et al., 2013).

Broiler producers commonly apply chemical amendments to improve litter conditions, reduce moisture, suppress pathogen proliferation, and limit ammonia (NH_3) volatilization. Acidifying agents such as sodium bisulfate ($NaHSO_4$) are widely used to lower litter pH, thereby reducing the conversion of nonvolatile ammonium (NH_4^+) into volatilized NH_3 (Ritz et al., 2004; Rothrock et al., 2010). Although the effectiveness of $NaHSO_4$-based amendments in reducing NH_3 emissions is well established (Johnson et al., 2011), their impacts on litter microbiome structure and composition remain less understood (Toppel et al., 2019; de Toledo et al., 2020; Johnson et al., 2021). While pH reduction is associated with a general decrease in total bacterial load (Johnson et al., 2021 and references therein), some evidence suggests that it may inadvertently promote the persistence of potentially pathogenic bacterial taxa, including *Salmonella* (Williams et al., 2012) and *Escherichia* (Joerger et al., 2020; Johnson et al., 2021). This phenomenon may be attributed to physiological characteristics such as acid tolerance and nitrogen metabolism, allowing select organisms to survive under lowered pH conditions and reduced competitive pressures resulting from microbial load decline (Bucher et al., 2020; Johnson et al., 2021; Swelum et al., 2021). The potential for dysbiosis following acidification highlights the need to investigate litter microbiome dynamics more holistically (e.g., via metabarcoding approaches) and suggests that complementary interventions may be warranted to preserve microbiome stability while optimizing NH_3 reduction to support bird health and welfare.

Biological amendments, including prebiotics, probiotics, and postbiotics, are increasingly employed in poultry production systems to reduce reliance on antibiotic growth promoters (Ricke 2018; Callaway and Ricke, 2023). These technologies are formulated primarily as feed or water additives (Pedroso et al., 2013; Swanson et al. 2025) and have been shown to elevate mutualistic microorganisms that competitively exclude pathogens in the gastrointestinal tract, directly suppress pathogen proliferation through specialized metabolites or antimicrobial compounds, prime the host immune system, and improve production outcomes by enhancing nutrient uptake, digestibility, and intestinal integrity (Reuben et al., 2021). However, the application of biological amendments to poultry litter remains comparatively underexplored relative to their use as ingestible interventions (Pedroso et al., 2013), and much of the existing literature reflects controlled microcosm conditions that may not fully capture microbial dynamics in commercial production environments (Johnson et al., 2021). Expanding biological intervention strategies to the litter environment is particularly relevant given that poultry litter harbors zoonotic pathogens,

including *Salmonella*, *Campylobacter*, and *Staphylococcus* species, which are prominently associated with the acquisition and dissemination of antimicrobial resistance (Gilchrist et al., 2007). Thus, biological amendments designed for litter application may offer a novel opportunity to enhance both food safety and bird performance.

IndigoLT™ (LA-I, AgriGro, Inc.) is a fermentation-based biotic litter amendment formulated to support microbial stability in poultry litter. The biotic-based technology contains a prebiotic/postbiotic fraction composed of organic acids, hygroscopic sugar alcohols, nitrogenous compounds, and antimicrobial compounds/specialized metabolites derived from a proprietary microbial consortium. In addition to the biological fraction, LA-I includes liquid $CaCl_2$ formulated with additional organic acid chelation. Although its commercial application is expanding, the effects of LA-I on litter microbial community structure and ecological function remain largely unexplored.

This preliminary study aimed to evaluate the effects of LA-I litter amendment on the prokaryotic litter microbiome in a commercial broiler operation. Litter samples were collected from three poultry houses: one treated only with $NaHSO_4$ (control), one treated with LA-I for a single flock, and one treated across two consecutive flocks. Both LA-I regimens included reduced rates of $NaHSO_4$. Microbial communities were characterized using 16S rRNA gene sequencing targeting the V4 region, and shifts in diversity, composition, and taxon abundance were assessed. Findings from this study provide initial insight into how different litter amendments may modulate microbial ecology and support the development of microbiome-targeted strategies for sustainable poultry production.

Materials and Methods

Study design

The test site was identified at Gillham, Arkansas, USA (34.16214°N, 94.35262°W) and encompassed three commercial broiler houses with 500 X 43 ft dimensions (21,500 ft²; ≈ 1,997 m²), selected for their consistent history of litter management and bird productivity. The bedding material, which consisted of locally sourced rice (*Oryza sativa*) hulls, had been used for 18 consecutive broiler flocks prior to study initiation, with a cake layer removed between each flock. The houses were managed under a mechanical ventilation system, with fans operating on a 300-s cycle for a minimum of 60 s at the beginning of the flock and increasing to ≈ 240 s by market weight (≈ 52 d). Temperature was initiated at 89-90 °F (31.7-32.2 °C) and was gradually reduced to 65-68 °F (18.3-20.0 °C) as the birds approached market size, while relative humidity was maintained at approximately 50-70%. Feed composition followed a standard four-phase broiler regimen: Starter (d 1-17), Grower (d 17-37), WD1/Finisher (d 36-47), and WD2/Finisher (d 48-onward). Two houses (denoted hereafter as "1" and "2") received the prebiotic amendment IndigoLT ("LA-I"; AgriGro Inc, Doniphan, MO, USA) at a rate of 48 oz/1,000 ft² (≈ 1.47 L/93 m²) 72 hrs before chick placement. Treatment 2 also received IndigoLT during the preceding flock (**Table 1**). Prebiotic applications were made with a spot sprayer between litter windrowing and leveling.

116 Approximately 16,333 Ross 308 chicks (Aviagen, Huntsville, AL, USA) were placed per
117 house, corresponding to a stocking density of 1.32 birds/ft² (8.18 birds/m²). Less than 24 hours
118 prior to chick placement, an $NaHSO_4$-based litter amendment (Jones-Hamilton Co., Maumee, OH,
119 USA) was applied to houses 1 and 2 at a rate of 40 lbs/1,000 ft² (≈ 195 g/m²) and to house 3
120 ("Control") at 100 lbs/1,000 ft² (≈ 488 g/m²) (**Table 1**). All other production parameters remained
121 constant across treatments.
122

Table 1 Litter amendment regimen for each treatment

Treatment	LA-I	$NaHSO_4$
1	1.47 L/93 m² (1st exposure)	195 g/m²
2	1.47 L/93 m² (2nd exposure)	195 g/m²
Control	——	488 g/m²

123
124 Sample collection and DNA isolation
125 On the day following bird removal, six composite litter samples (n = 18) were collected
126 from each house to capture spatial heterogeneity. Each composite consisted of five subsamples
127 collected to a depth of 10 cm using a sanitized trowel, with a new trowel used for each house to
128 prevent cross-contamination. Subsamples were pooled into sterile ziplock bags and immediately
129 flash-frozen on dry ice. Samples were then shipped on dry ice to the University of Missouri (MU)
130 Genomics Technology Core, where they were stored at −80 °C until DNA isolation.
131 DNA was isolated using the QIAamp PowerFecal Pro DNA Kit (Cat. #51804; Qiagen,
132 Hilden, Germany) according to the manufacturer's instructions, except that samples were
133 homogenized using a TissueLyser II (Qiagen) at 30 Hz for 10 minutes in place of the vortex adapter
134 specified in the protocol. DNA concentrations were determined fluorometrically using a Qubit 2.0
135 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with the Quant-iT dsDNA Broad-
136 Range Assay Kit (Cat. #Q33130) and subsequently normalized to a consistent concentration and
137 volume.
138
139 16S rRNA library preparation and sequencing
140 Library preparation and sequencing were conducted at the MU Genomics Technology
141 Core. Prokaryotic 16S rRNA amplicons were generated by targeting the V4 region of the 16S
142 rRNA gene using universal primers U515F/806R, flanked by Illumina standard adapter sequences
143 (Caporaso et al., 2011; Walters and Caporaso et al., 2011). All reactions employed dual-indexed
144 forward and reverse primers. PCR was performed in 50 µL reactions containing 100 ng DNA,
145 0.2 µM of each primer, 200 µM of each dNTP, 1 U of Phusion High-Fidelity DNA Polymerase
146 (Cat #F530S; Thermo Fisher Scientific), and the appropriate volume of 5X Phusion HF Buffer
147 (Cat. #F518L; Thermo Fisher Scientific). Amplification conditions included initial denaturation at

98 °C for 3 min, followed by 25 cycles of 98 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min.

Following amplification, 5 µL of product from each reaction was pooled, mixed thoroughly, and purified using Axygen Axyprep MagPCR Clean-up Beads (Cat. #14-223-227; Thermo Fisher Scientific) at a 1:1 bead-to-amplicon volume ratio. The mixture was incubated for 15 min at room temperature, washed twice with 80% ethanol, and air-dried. The purified pellet was resuspended in 32.5 µL of EB buffer (Cat. #19086; Qiagen), incubated at room temperature for 2 min, and placed on a magnetic stand for 5 min to recover the supernatant.

The final pooled amplicon library was evaluated using the Advanced Analytical Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA), quantified with the Quant-iT High Sensitivity dsDNA Assay Kit (Cat. #Q33120; Thermo Fisher Scientific), and diluted according to Illumina's protocol for sequencing on the MiSeq platform as 2 × 250 bp paired-end reads.

Bioinformatic analysis

DNA sequences were assembled and annotated at the MU Bioinformatics and Analytics Core. Primers were designed to match the 5′ ends of the forward and reverse reads. Adapter and primer removal was conducted using Cutadapt (v2.6) (Martin, 2011). The forward primer was trimmed from the 5′ end of the forward read, and, if present, the reverse complement of the reverse primer was removed from the 3′ end of the same read along with all downstream bases, resulting in bidirectional trimming if the insert was shorter than the amplicon. The reverse read was processed analogously, with primer roles reversed. Read pairs were discarded if either read failed to match a 5′ primer, with an allowed error rate of 0.1 and a minimum overlap of 3 bp at the 3′ end of the primer for removal. Each read underwent two passes to ensure complete primer removal.

Amplicon sequence variant (ASV) inference was performed using the DADA2 plugin (v1.30.0) (Callahan et al., 2016) within QIIME2 (v2024.5.1) (Bolyen et al., 2019). Denoising parameters included truncating both forward and reverse reads to 150 bases, discarding reads with more than 2.0 expected errors, and removing chimeras using the 'consensus' method. The QIIME2 environment incorporated Python (v3.9.19) and BIOM (v2.1.15) for downstream processing. Taxonomic classification was performed using the 'classify-sklearn' method with the SILVA v138.1 reference database (Quast et al., 2012).

Statistical analysis

The QIIME2 artifacts were imported into RStudio (v4.4.0) (R Core Team, 2020) with the qiime2R package (v0.99.6) (Bisanz et al., 2018) and were used to create a "phyloseq" S4 object (v1.48.0) (McMurdie and Holmes, 2013). One sample with <1,000 reads was removed prior to analysis. Per-sample coverage/sequencing depth was then estimated using Good's coverage, calculated with the 'phyloseq_coverage' function from the metagMisc package (v0.5.0) (Mikryukov, 2020) with the formula $1 - (F1/N)$, where $F1$ is the number of singleton ASVs and N is the read sum for all ASVs (Good, 1953; Good and Toulmin, 1956; Chao and Jost, 2012). Observed ASVs (richness) were evaluated as a function of sequencing depth using the 'rarecurve'

function from the vegan package (v2.6.1) (Dixon, 2003). All visualizations, including family-level relative abundance plots, were generated with ggplot2 (v3.4.2) (Wickham and Sievert, 2009). The rooted phylogenetic tree was converted to a "phylo" object using the ape package (v5.8) (Paradis et al., 2004) and visualized as a cladogram with ggtree (v3.12.0) (Yu et al., 2017).

Within-sample (α) diversity was calculated after rarefying all samples to the minimum sequencing depth (170,641 reads per sample). Richness and Shannon diversity (Hill, 1973) were determined with the 'estimate_richness' phyloseq function, Pielou's evenness (Pielou, 1966) with the 'evenness' function from the microbiome package (v1.26.0) (Lahti and Shetty, 2018), and Faith's phylogenetic diversity (Faith, 1992) with the 'pd' function in picante (v1.8.2) (Kembel et al., 2010). For each metric, Levene's test (Levene, 1960) was used to assess homogeneity of variance across treatments via the 'leveneTest' function in the car package (v3.1-2) (Fox et al., 2012), and the Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to evaluate normality within each treatment group with 'shapiro.test' from the stats package (v4.4.0). All assumptions of homogeneity and normality were met ($p > 0.05$), and treatment effects were evaluated using analysis of variance (ANOVA) models fitted with the 'aov' function in stats. The α diversity estimates were visualized with ggplot2.

Prior to β diversity analysis, reads were normalized using cumulative sum scaling (CSS) with the 'cumNormStat' and 'cumNorm' functions from the metagenomeSeq package (v1.46.0) (Paulson et al., 2013). Global dissimilarity matrices were then constructed using Bray-Curtis (Bray and Curtis, 1957), Jaccard (1908), Weighted UniFrac, and Unweighted UniFrac (Lozupone and Knight, 2005) distances via the 'distance' function in phyloseq. Ordination was performed using Principal Coordinates Analysis (PCoA) with the 'ordinate' function in phyloseq, and results were visualized with ggplot2. Compositional dissimilarity among treatment levels was assessed statistically using permutational multivariate analysis of variance (PERMANOVA) with the 'adonis2' function from vegan (v2.6-6.1) with 9,999 permutations. As PERMANOVA identified significant treatment effects ($p < 0.05$) for all distance metrics, multilevel pairwise comparisons were performed using 'pairwise.adonis' from the pairwiseAdonis package (v0.4.1) (Martinez Arbizu, 2020), applying false discovery rate (FDR) correction to control for multiple testing. Homogeneity of multivariate dispersions was evaluated with the 'betadisper' function from vegan, and group centroid distances were compared across treatment levels using a permutational ANOVA model implemented with the 'anova' function from the stats package (9,999 permutations).

Global and treatment-specific core microbiome features were defined as ASVs present in all but one sample per condition, following the "relaxed core" concept proposed by Mejia et al. (2025). Per-sample relative abundances of core ASVs were calculated from CSS-normalized counts using the 'transform_sample_counts' function in phyloseq, and visualized with the ComplexHeatmap package (v2.20.0) (Gu et al., 2016). The top 10 most represented genera, families, and orders by ASV count within each core microbiome were identified and visualized using ggplot2. Shared and exclusive ASVs among core groups were further visualized using the

ggvenn package (v0.1.10) (Yan, 2021). Core microbiomes were clustered using Jaccard distance, calculated with the 'vegdist' function from the vegan package and visualized in ggplot2.

Features differentially abundant among treatment levels were identified using the MaAsLin2 package (Microbiome Multivariable Associations with Linear Models) (v1.18.0) (Mallick et al., 2021). Log-transformed linear models were constructed using total sum scaling (TSS)-normalized count data following MaAsLin2 default parameters. Pairwise treatment comparisons were performed independently. To reduce the influence of spurious or environmentally stochastic features in the absence of a shared baseline, analyses were restricted to features present in the core microbiomes of at least two treatment groups. Genus-, family-, and order-level counts were additionally generated using the 'tax_glom' function from the phyloseq package and reanalyzed using the same MaAsLin2 parameters. Features with a Benjamini-Hochberg-adjusted *p*-value ($q < 0.25$) (Benjamini and Hochberg, 1995) were considered differentially abundant as recommended by MaAsLin2 documentation (Mallick et al., 2021). The log-normalized FDR [−sign(coefficient) × log(*q*-value)] (Boolchandani et al., 2022), which is equivalent to $\log_2$(fold change) (Reasoner et al., 2024), was calculated for significant features and visualized using the ComplexHeatmap package. Shared and unique features across pairwise comparisons were visualized with the ggvenn package.

Results

Sequencing data overview

The objective of the present study was to determine the impacts of LA-I, a commercial amendment, on poultry litter microbiome dynamics when integrated into a conventional litter management system. Comparisons were made between a house receiving LA-I plus 40% of the recommended $NaHSO_4$ application for the first time (treatment 1), a house treated with the same combination for a second consecutive flock (treatment 2), and a control house receiving only the recommended $NaHSO_4$ application. Six composite litter samples were collected per house, and the V4 region of the 16S rRNA gene was amplified and sequenced to assess prokaryotic microbiome shifts across treatments.

Illumina V4 sequencing yielded 333,596 ± 48,470 reads per sample (mean ± 1 SD), with Good's coverage estimates (0.999994 ± 6e–6) and rarefaction curves supporting adequate sampling depth (**Figure 1a**–**c**). Denoising rendered 2,938 ASVs, with individual samples encompassing 531.94 ± 157.88. The dominant phyla were Firmicutes (59.87% of reads, 69.8% of ASVs), Actinobacteriota (35.95% of reads, 11.37% of ASVs), and Proteobacteria (3.31% of reads, 7.26% of ASVs). The most abundant classes were Bacilli (54.58% of reads, 25.61% of ASVs), Actinobacteria (35.94% of reads, 10.51% of ASVs), and Clostridia (5.27% of reads, 43.48% of ASVs). Dominant orders included Staphylococcales (30.48% of reads, 4.16% of ASVs), Micrococcales (19.80% of reads, 5.92% of ASVs), and Lactobacillales (17.74% of reads, 7.84% of ASVs). At the family level, Staphylococcaceae (30.48% of reads, 4.16% of ASVs), Dermabacteraceae (10.01% of reads, 1.10% of ASVs), and Lactobacillaceae (9.40% of reads, 3.30% of ASVs) were most prevalent (**Figure 1d**). Dominant families were distributed in

taxonomically consistent clades within a rooted phylogenetic tree, reflecting anticipated sequence homology (**Figure 1e**).

LA-I administration influenced prokaryotic microbiome composition

Treatment effects on α diversity were evaluated using mean estimates derived from 1,000 rarefied iterations for Faith's phylogenetic diversity, observed ASVs, Shannon diversity, and Pielou's evenness. No statistically significant differences were detected among treatments for any metric (ANOVA, $p > 0.05$) (**Figure 2**). Following α diversity analysis, count matrices were normalized via cumulative sum scaling (CSS), and β diversity was assessed to infer treatment-driven compositional shifts.

Despite the absence of significant α diversity differences, treatment exerted a significant effect on microbial community composition (PERMANOVA, $p < 0.05$) across all tested dissimilarity measures, including Bray–Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac distances (**Figure 3**). These global results warranted pairwise comparisons to identify specific treatment-level differences. Compositional differences were detected between treatment 1 and control across all distance metrics except

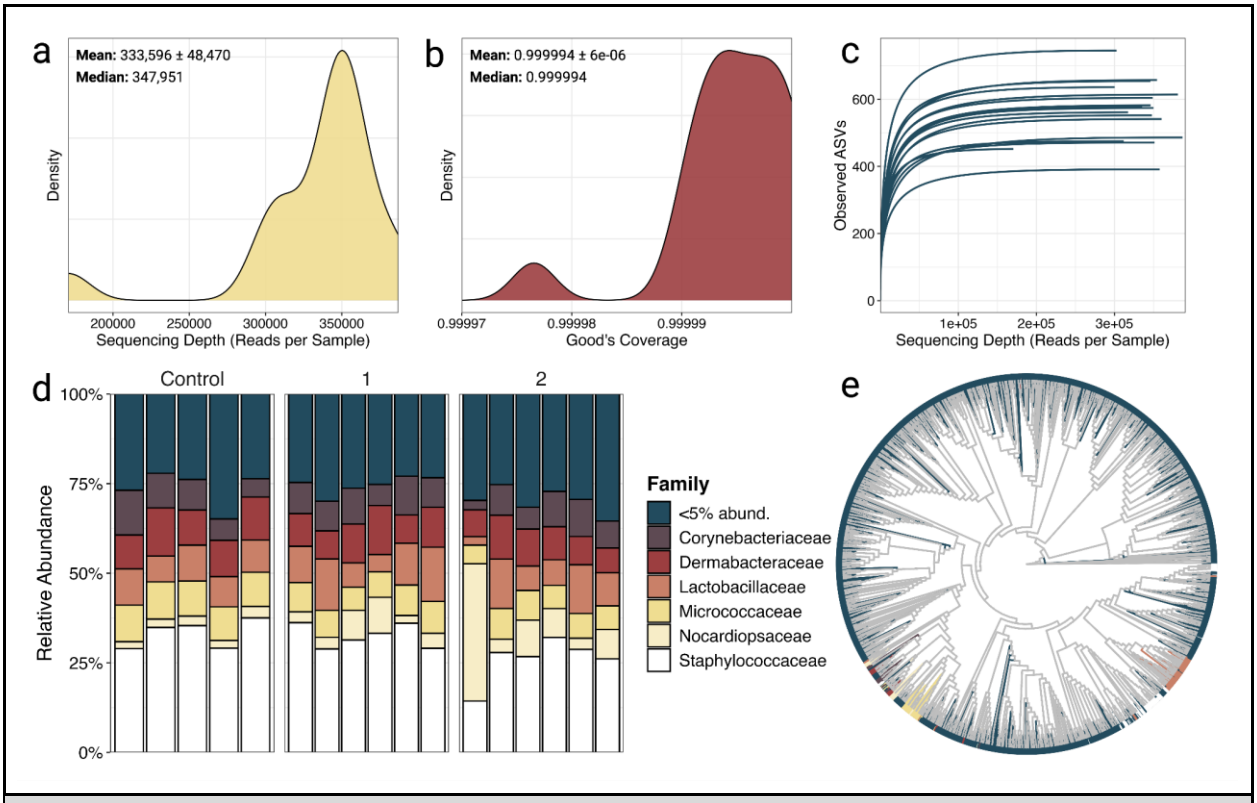

Figure 1 Overview of the poultry litter microbiome. (**a**) Sequencing depth distribution. (**b**) Distribution of Good's coverage determined with the formula: *1 - (F1/N)*, where *F1* is the number of singleton OTUs and *N* is the read sum for all OTUs. (**c**) Rarefaction curve showing observed ASVs (richness) as a function of sequencing depth (reads per sample). (**d**) Stacked bar plot of family-level per-sample composition. Families contributing less than 5% of total read abundance across all samples were grouped into "<5% abund." after agglomerating counts to the

Family level. (**e**) Rooted phylogenetic tree. Tip color corresponds to family utilizing the same key as the stacked bar plot.

Weighted UniFrac (q = 0.29, R^2 = 0.15) (**Figure 3c**), and between treatment 2 and control across all metrics (**Figure 3**). No significant differences were observed between treatments 1 and 2 (q > 0.05), suggesting that the LA-I regimen, regardless of duration of use, may have consistently altered the litter microbiome composition relative to the control. These compositional shifts were not attributable to differences in within-group variation, as global estimates of β dispersion were statistically insignificant (ANOVA, p > 0.05) for all distance metrics (**Figure 3**) (Anderson et al., 2006).

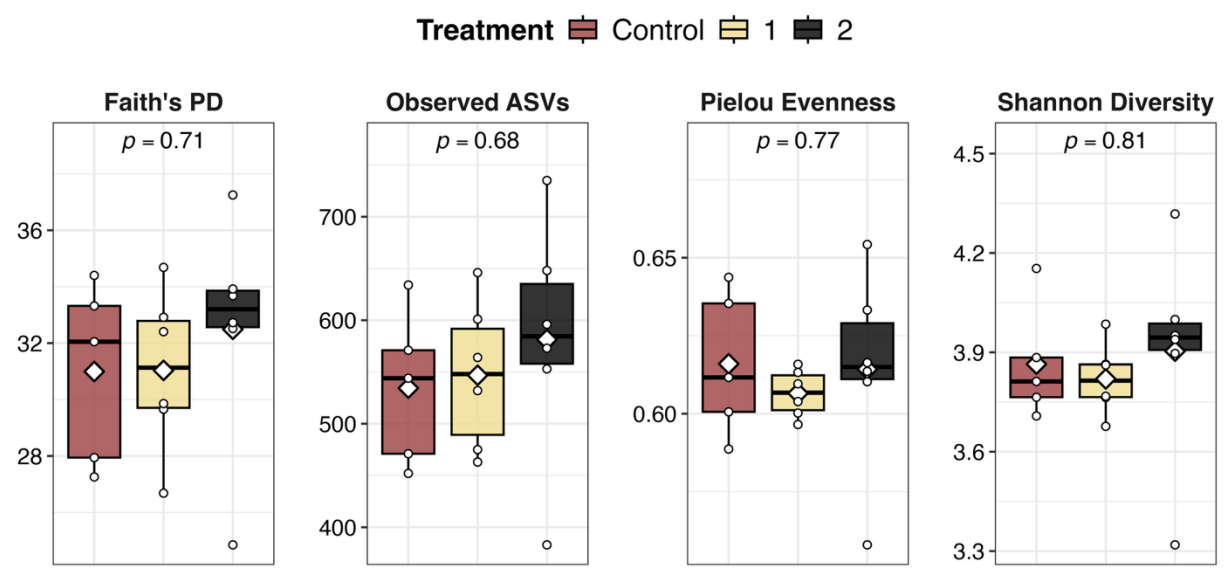

Figure 2 Estimation of α diversity with Faith's phylogenetic diversity, observed ASVs (richness), Pielou's evenness, and Shannon diversity. Values reflect diversity metrics computed on libraries rarefied to 170,641 reads per sample (minimum depth). Shapiro-Wilks tests were performed to determine if values for each treatment-metric combination were normally distributed, and Levene's test was used to discern if variance was homogenous between treatments for each metric. Mean separation was performed with ANOVA.

Treatment altered core microbiome membership without impacting global diversity

Given the perceived role of the core microbiome in promoting ecological stability (Neu et al., 2021; Jiao et al., 2022), core taxa were identified as ASVs present in at least all but one sample across the collective dataset (Mejia et al., 2025). A total of 170 ASVs were detected in the global core microbiome (**Figure 4a**, **d**). The most represented genus was *Christensenellaceae_R-7_group* (6 ASVs), followed by eight genera each represented by four ASVs (**Figure 4c**). The most prevalent families included Lachnospiraceae (19 ASVs), Bacillaceae (16 ASVs), and

Oscillospiraceae (14 ASVs), while dominant orders included Oscillospirales (31 ASVs), Lactobacillales (25 ASVs), and Micrococcales (21 ASVs) (**Figure 4c**).

Treatment-specific core microbiomes were subsequently characterized, revealing distinction in ASV presence with LA-I regimens. Across all treatments, 334 core ASVs were identified, representing the 170 ASVs shared globally and 164 that were treatment specific (**Figure 4a**–**b**, **d**). Treatment 1 shared 215 of 334 (64.37%) core ASVs with the control, while treatment 2 shared 184 of 334 (55.09%) (**Figure 4d**). Treatments 1 and 2 shared 189 of 334 (56.59%) core ASVs (**Figure 4d**). Forty-eight ASVs were unique to the control core microbiome, including ASVs mapped to *Faecalibacterium* and *Nocardiopsis salina* (**Figure 4b**). Twenty-three ASVs were unique to treatment 1 and fifteen to treatment 2 (**Figure 4b**). Nineteen ASVs were shared between treatments 1 and 2 but absent from the control, such as *Lactobacillus aviarius* and *Bifidobacterium pullorum* (**Figure 4b**). Clustering of ASVs based on Jaccard distances supported these findings, with greater dissimilarity observed between the control and treatment 2 relative to the control and treatment 1 (**Figure 4e**).

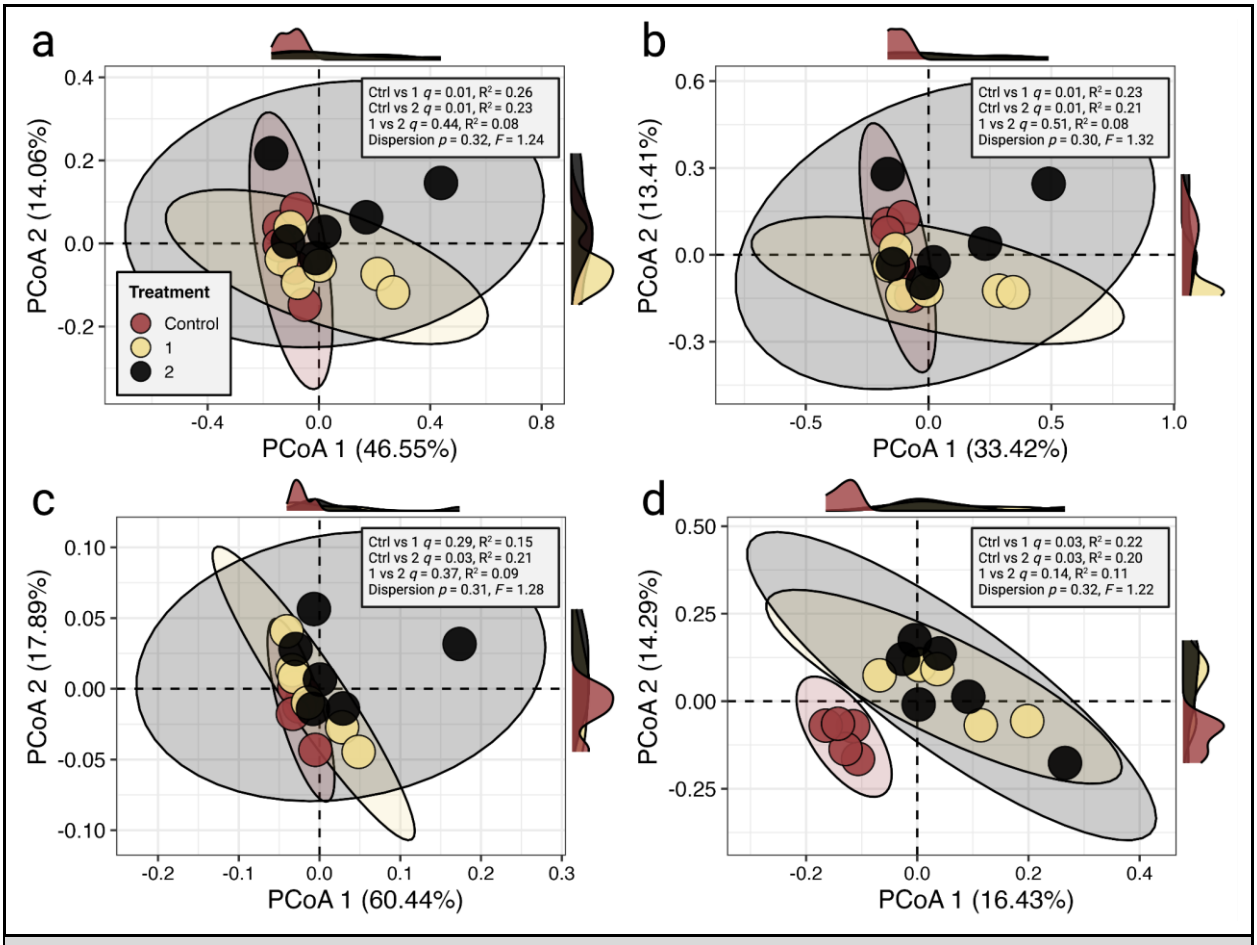

Figure 3 β diversity assessment leveraging (**a**) Bray-Curtis, (**b**) Jaccard, (**c**) Weighted UniFrac, and (**d**) Unweighted UniFrac distance matrices. Due to statistical significance with global PERMANOVA ($p < 0.05$), pairwise PERMANOVA was used to identify compositional

dissimilarity between treatments. Pairwise PERMANOVA and global ϭ dispersion results are in the top right corner of each plot.

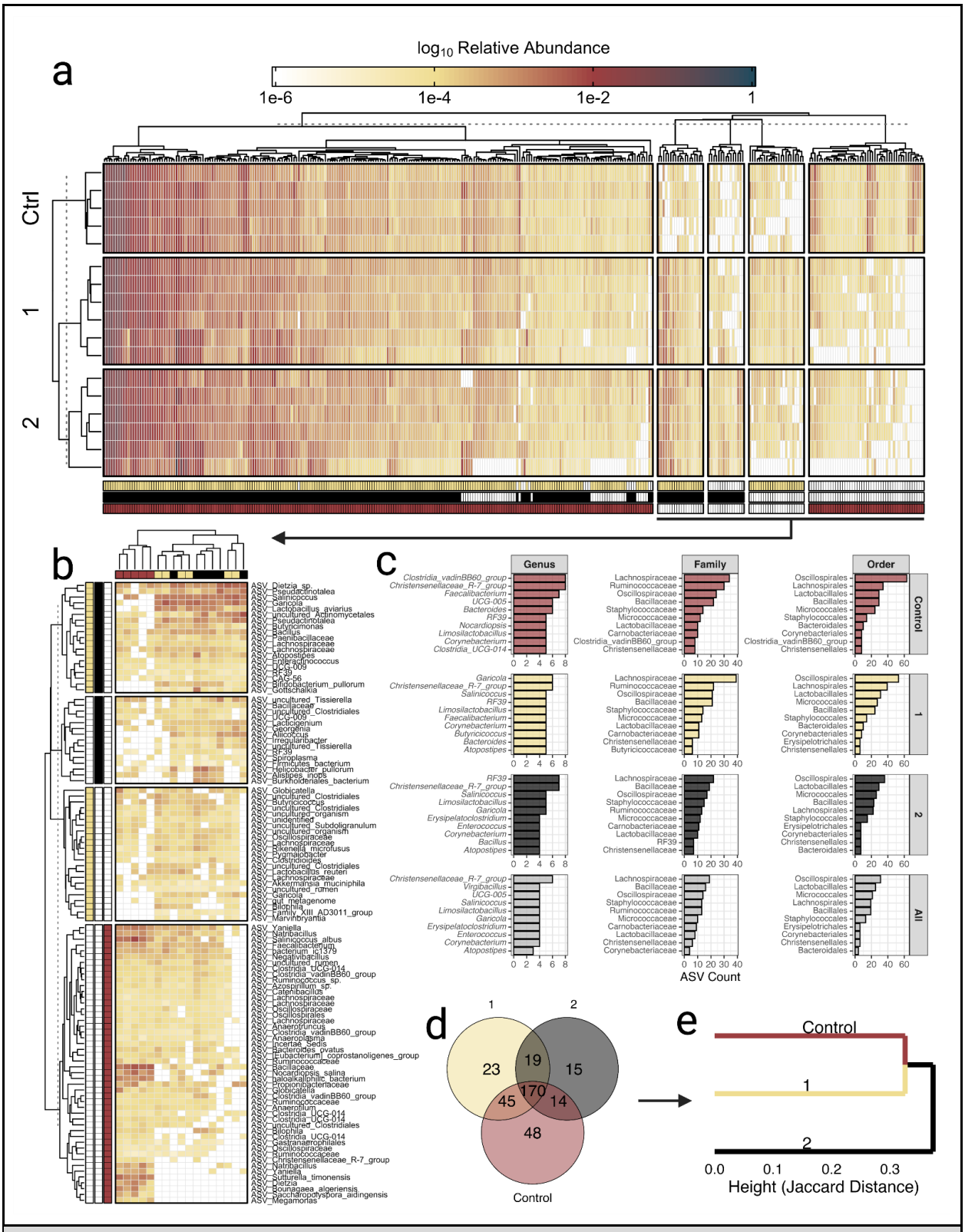

Figure 4 Global and treatment-specific core microbiome analysis. (**a**) Relative abundance ($\log_{10}$) heatmap of ASVs represented in one or more core microbiomes. Column annotations reflect

representation in control (red), 1 (yellow), and 2 (black) treatments. (**b**) Relative abundance heatmap of ASVs represented exclusively in control, 1, 2, or 1 and 2 (yellow and black row annotations). (**c**) Top ten genera, families, and orders represented in global and treatment-specific core microbiomes. (**d**) Venn diagram depicting shared and exclusive ASV counts across the treatment-specific core microbiomes. (**e**) Clustering of treatment-specific core microbiomes with Jaccard distances.

At the genus level, shifts in core microbiome composition were evident across treatments. In the control, the most dominant genera included *Clostridia_vadinBB60_group* and *Christensenellaceae_R-7_group* (8 ASVs each), followed by *Faecalibacterium*, *UCG-005*, and *Bacteroides* (7, 6, and 6 ASVs, respectively) (**Figure 4c**). Additional genera such as *RF39*, *Nocardioides*, *Limosilactobacillus*, and *Corynebacterium* were also present, reflecting a diverse community enriched in Firmicutes and Actinobacteria (**Figure 4c**). In treatment 1, *Garciella* and *Christensenellaceae_R-7_group* (6 ASVs each) were the most abundant, followed by eight genera represented by five ASVs each (**Figure 4c**). Treatment 2 was composed primarily of *Christensenellaceae_R-7_group* and *RF39* (7 ASVs each), along with *Garicola*, *Limosilactobacillus*, and *Salinicoccus*, each represented by five ASVs (**Figure 4c**). Treatment-specific core microbiomes displayed similar compositions at the family and order levels (**Figure 4c**).

<u>Differential abundance analysis revealed consistent taxonomic shifts following LA-I treatment</u>

Differential abundance testing was performed at ASV level using log-transformed linear models, with the control treatment specified as a reference. A total of 115 ASVs were differentially abundant ($q < 0.25$) between the LA-I regimens and the control, with 82 identified between treatment 1 and the control (39 enriched, 43 depleted) and 81 between treatment 2 and the control (45 enriched, 36 depleted). Among these, 48 of 115 (41.74%) exhibited consistent directional changes in response to the LA-I application, with 23 enriched and 25 depleted.

To assess the biological relevance of these shifts, abundance matrices were agglomerated at higher taxonomic ranks and reanalyzed using MaAsLin2. Because incomplete taxonomic assignment can bias interpretation at broader ranks, the proportion of ASVs and reads mapped to each taxonomic level was calculated. Taxa were considered assigned if annotations were non-missing and excluded ambiguous terms such as “unknown,” “uncultured,” “uncultured_bacterium,” or “uncultured organism,” consistent with the logic applied to heatmap and Venn diagram visualizations. Based on these criteria, 100% of ASVs were assigned at the kingdom level, followed by 99% at phylum and class, 97% at order, 93% at family, 73% at genus, and 15% at species. Read-level assignments followed a similar trend, with 100% of reads mapped through the family level, 91% at genus, and 22% at species (**Supplemental Figure 1**). Therefore, order-, family-, and genus-level agglomerations were selected for secondary differential abundance testing.

At the genus level, 20 features were differentially abundant in one or both LA-I treatments relative to the control (**Figure 5a**). Of these, 10 were detected in treatment 1 vs. control (6 enriched, 4 depleted) and 19 in treatment 2 vs. control (16 enriched, 3 depleted), with nine of 20 (45%) shared and showing consistent directional changes (6 enriched, 3 depleted). Genera enriched in both treatments included *Bifidobacterium* (1 vs. ctrl: coef = 3.56, q = 0.04; 2 vs. ctrl: coef = 2.26, q = 0.01) and *Brevibacterium* (1 vs. ctrl: coef = 1.10, q = 0.04; 2 vs. ctrl: coef = 1.09, q = 0.14), while *Staphylococcus* was consistently depleted (1 vs. ctrl: coef = –2.68, q = 0.16; 2 vs. ctrl: coef = –2.44, q = 0.11). The genus-level trend unique to treatment 1 was the depletion of *Campylobacter* (coef = –5.33, q = 9e-5), while those exclusive to treatment 2 included enrichment of *Atopostipes* (coef = 0.89, q = 0.23), *Halomonas* (coef = 2.38, q = 0.11), *Nocardiopsis* (coef = 1.69, q = 0.14), *Oceanobacillus* (coef = 0.85, q = 0.14), and nine additional genera (**Figure 5a**).

Family-level analysis identified 15 differentially abundant features, with 10 in treatment 1 vs. control (6 enriched, 4 depleted) and 14 in treatment 2 vs. control (11 enriched, 3 depleted). Nine of 15 (60%) were shared and showed consistent directional changes (6 enriched, 3 depleted), largely mirroring genus-level trends (**Figure 5a**). Consistent shifts included the enrichment of Bifidobacteriaceae (1 vs. ctrl: coef = 3.55, q = 0.02; 2 vs. ctrl: coef = 2.25, q = 0.005), Brevibacteriaceae (1 vs. ctrl: coef = 1.16, q = 0.02; 2 vs. ctrl: coef = 1.15, q = 0.1), and four additional families, and the depletion of Marinococcaceae (1 vs. ctrl: coef = -4.05, q = 0.005; 2 vs. ctrl: coef = -3.94, q = 0.01), Micrococcaceae (1 vs. ctrl: coef = -0.35, q = 0.02; 2 vs. ctrl: coef = -0.51, q = 0.04), and Sutterellaceae (1 vs. ctrl: coef = -3.21, q = 0.01; 2 vs. ctrl: coef = -2.71, q = 0.1). Furthermore, the treatment 1 vs. control comparison saw the depletion of Campylobacteraceae (coef = –5.26, q = 5.3e-5), while treatment 2-specific enrichments included Actinomycetaceae (coef = 1.79, q = 0.1), Bogoriellaceae (coef = 1.57, q = 0.21), Carnobacteriaceae (coef = 1.0, q = 0.11), Nocardiopsaceae (coef = 1.76, q = 0.1), and Paenibacillaceae (coef = 1.37, q = 0.12) (**Figure 5a**).

At the order level, 14 features were differentially abundant across comparisons. Treatment 1 vs. control accounted for 11 orders (7 enriched, 4 depleted), while 9 were detected in treatment 2 vs. control (7 enriched, 2 depleted). Six of 14 (42.86%) were shared and showed consistent directional change (5 enriched, 1 depleted), with most patterns reflecting shifts observed at lower ranks (**Figure 5a**).

Consistent with β diversity trends, treatments 1 and 2 exhibited broadly similar differential abundance profiles relative to the control. Specifically, 24 of 48 (50%) genus-to-order features (excluding unannotated genera) and 72 of 164 (43.9%) total differentially abundant features overlapped between comparisons (**Figure 5b**,**c**), with all shared features (72/72) showing consistent directional change (**Figure 5b**,**c**). Notably, 41 of 164 (25%) and 51 of 164 (31.1%) features were unique to treatment 1 vs. control and treatment 2 vs. control, respectively, indicating potential distinctions between early and sustained exposure to the LA-I regimen (**Figure 5b**,**c**).

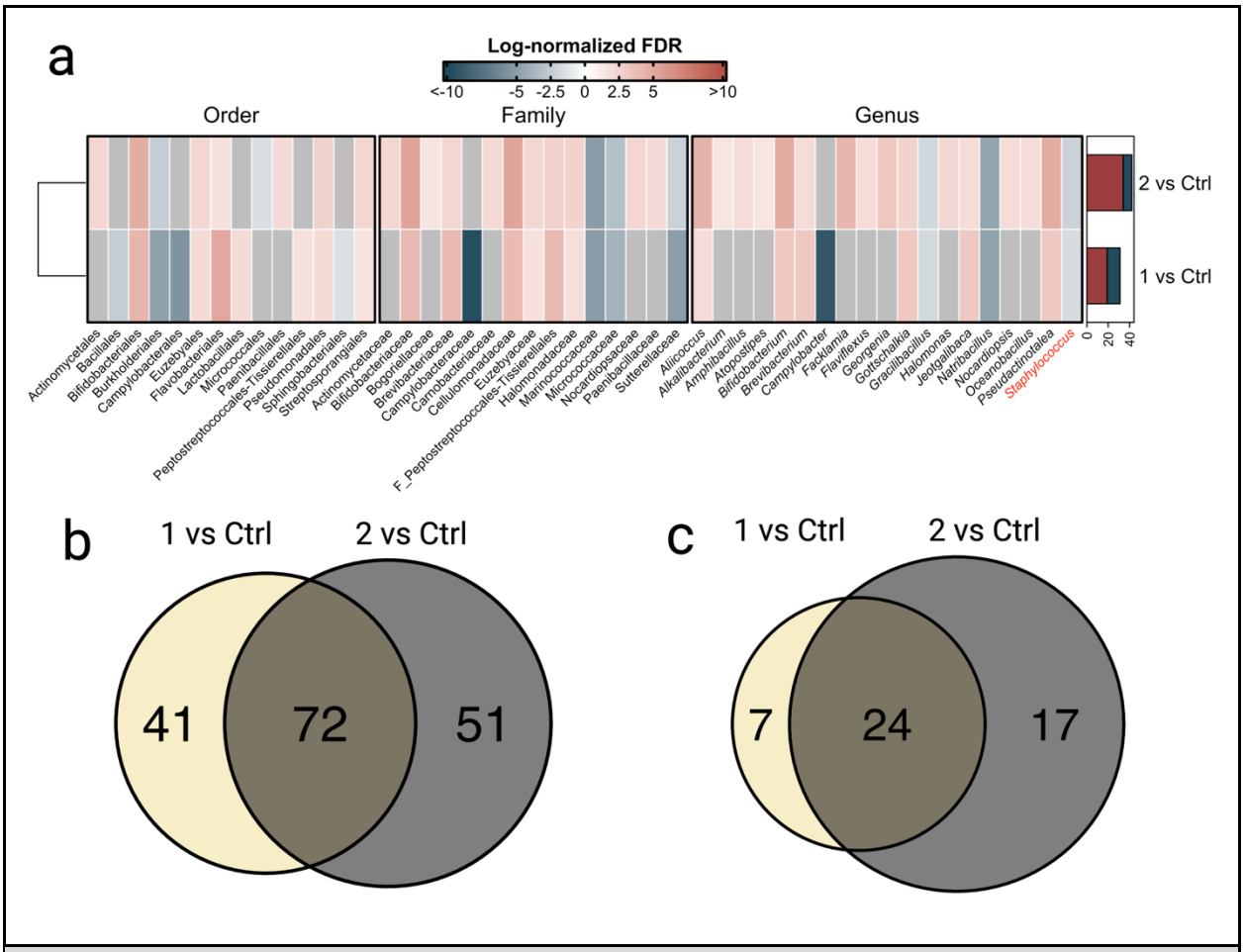

Figure 5 Differential abundance testing of LA-I vs. control treatments. (**a**) Heatmap of differentially abundant features ($q \leq 0.25$) identified at genus, family, and order levels using a log-transformed linear model. (**b**) Venn diagram of all shared and distinct differentially abundant features among treatment comparisons. (**c**) Venn diagram excluding ASVs and genera without taxonomic information (only features in the heatmap).

Discussion

<u>LA-I application restructured the broiler litter microbiome</u>

This work demonstrates how LA-I application, in conjunction with reduced $NaHSO_4$, may alter the structure and composition of the broiler litter microbiome in a commercial setting. Despite no statistically significant differences in α diversity metrics (i.e., richness, evenness, phylogenetic diversity), β diversity analyses revealed that LA-I treatments yielded comparable community profiles that were distinct from the control across all dissimilarity measures. These results align with Do et al. (2024), who reported minimal effects of an organic litter amendment on α diversity despite improved flock performance. In contrast, Johnson et al. (2021) observed increased richness shortly after $NaHSO_4$ application, but found no major changes to bacterial community structure unless topdressing was also applied. The absence of significant differences between the two LA-I

regimens in the present study suggests that a single flock exposure may be sufficient to restructure the microbial community in a way that persists across subsequent production cycles.

The core microbiome is thought to encompass the most functionally important microbiota which interact synergistically/antagonistically with transient taxa (Neu et al., 2021; Jiao et al., 2022). In the present study, the core microbiome remained largely stable across treatments, although genus-level shifts became more pronounced with prolonged application of LA-I. The most represented genus in the global core was *Christensenellaceae_R-7_group*, a cellulolytic bacterium commonly detected in the gastrointestinal tract of livestock and previously linked to host metabolic traits in beef cattle (Zhang et al., 2025), pigs (Shang et al., 2022), and goats (Liu et al., 2019). *Christensenellaceae_R-7_group* was also identified as the second most abundant genus in broiler feces (Shi et al., 2023). However, findings on its role in poultry remain inconsistent. Higher abundance of *Christensenellaceae_R-7_group* has been associated with improved feed conversion and residual feed intake (He et al., 2023), yet negative associations have also been reported with body weight and average daily gain (Xu et al., 2025), alongside reductions in abundance following dietary supplementation with bio-fermented distillers grain (Xu et al., 2025), N-carbamylglutamate (Liu et al., 2025), and fungal-derived bioactive compounds (Shi et al., 2023). These findings suggest that the ecological function of *Christensenellaceae_R-7_group* may be context-dependent or shaped by interactions with other microorganisms. For instance, in studies where negative associations with growth were observed, similar patterns were also reported for ASVs assigned to *Clostridia_vadinBB60_group* and *Oscillospiraceae*, indicating possible co-occurrence dynamics (Xu et al., 2025). Further investigation using larger sample sizes and microbial co-occurrence network analysis (Röttjers and Faust, 2018) will be necessary to resolve the ecological role of *Christensenellaceae_R-7_group* in poultry litter microbial communities.

Various ASVs of potential biological relevance demonstrated exclusivity across treatment-specific core microbiomes. For example, those exclusively present in LA-I–amended groups included an ASV encoding the carbohydrate-degrading bacterium *Bifidobacterium pullorum* (Baseer et al., 2023), an ASV mapped to the family *Paenibacillaceae* (members of which have demonstrated probiotic potential in poultry [Wu et al., 2019; Ekim et al., 2020; Lee et al., 2025]), a *Bacillus* ASV (Grant et al., 2018), and an ASV mapped to *Butyricimonas*, a genus positively associated with broiler growth performance (Johnson et al., 2018; Kairmi et al., 2024). The genera *Salinicoccus* and *Atopostipes* were also more represented in the LA-I–amended core microbiomes, with the former being characteristic of reused poultry litter (Wang et al., 2016; Kubasova et al., 2022), and both being indicator taxa in active poultry compost (Wang et al., 2022) and represented in the broiler litter core microbiome of Johnson et al. (2021).

In contrast, the control core microbiome exhibited exclusivity of three *Clostridia UCG-014* ASVs, a group reported to improve yellow broiler performance and influence tryptophan metabolism (Dai et al., 2023), while also shown to be significantly enriched following *Campylobacter jejuni* challenge (Valečková et al., 2023). The latter pattern is consistent with that reported by Dai et al. (2023), where *Clostridia UCG-014* co-occurred with *Oscillospiraceae* and *Faecalibacterium*, both of which were more abundant in the control core microbiome in the present

study. Similarly, *Faecalibacterium* spp. were enriched in response to $NaHSO_4$ in the work of Johnson et al. (2021), further supporting trends observed here. The exclusion of *Clostridia UCG-014* and reduced abundance of *Clostridiales vadinBB60 group* in LA-I–amended cores aligns with prior findings showing that both taxa respond to *Campylobacter jejuni* infection (Yan et al., 2021; Ty et al., 2022; Valečková et al., 2023). Notably, *Campylobacter* genus-level depletion was also observed in the present study in treatment 1 relative to the control. Additional trends that warrant further investigation included the exclusivity of an ASV of the broiler probiotic *Lactobacillus reuteri* (Chai et al., 2023) in the treatment 1 core, an ASV mapped to *Helicobacter pullorum* (Abd El-Ghany, 2020) in the treatment 2 core, and the increased representation of *Nocardiopsis* in LA-I–amended cores, a genus known for the synthesis of bioactive compounds (Shi et al., 2022) and that is negatively associated with *Salmonella* abundance in poultry litter (Bucher et al., 2020). These findings collectively suggest that employed LA-I regimens modulate core microbiome composition, enriching taxa with potential probiotic or metabolic relevance while reducing groups associated with pathogen challenge. Future efforts incorporating baseline measurements are needed to validate core microbiome trends.

<u>Differentially abundant taxa reflected functional reorganization of the litter microbiome following LA-I amendment</u>

Differential abundance testing was performed at the ASV, genus, family, and order levels. The decision to agglomerate and analyze at higher taxonomic ranks, rather than infer patterns solely from ASV-level assignments (as done in core microbiome analysis), was guided by the proportion of ASVs and reads that could be confidently mapped at each rank and by recent benchmarking of 16S differential abundance workflows (Wallen et al., 2021; Nearing et al., 2022). To accentuate taxa most likely governed by deterministic selection rather than stochastic or transient signals (Hayashi et al., 2024), features were limited to those comprising the treatment-specific core microbiomes of at least two groups. This filtering ensured that analyses would focus on reproducible, treatment-responsive community members. The following section highlights genus-level patterns associated with LA-I regimens.

The LA-I–amended treatments exhibited consistent increases in *Jeotgalibaca*, a genus positively associated with body weight, feed intake, and peripheral immune function in broilers (Song et al., 2022). *Brevibacterium*, a common inhabitant of poultry litter microbiota (Dumas et al., 2011) and a marker of nutrient cycling, organic matter decomposition, and litter maturation (Wang et al., 2016; Peng et al., 2021; Kubasova et al., 2022), was also enriched. Similarly, *Gottschalkia* was consistently elevated following LA-I amendment. This purinolytic genus degrades avian uric acid to NH_3 under anaerobic conditions, thereby contributing to nitrogen mineralization and early-stage litter decomposition (Poehlein et al., 2017). While uric acid degradation is a significant contributor to NH_3 emissions in poultry litter, *Gottschalkia* is urease-negative and therefore does not contribute to urea hydrolysis, the enzymatic step directly responsible for the production of NH_3 from urea (Rothrock et al., 2010). Its persistence later in the production cycle may reflect the diminished efficacy of $NaHSO_4$ over time (Hunolt et al., 2015),

as litter pH rebounds and conditions become more favorable for uricolytic activity. Moreover, enrichment of *Gottschalkia* may be linked to reduced $NaHSO_4$ input in LA-I treatments (relaxing the acid-mediated suppression of uricase-active taxa) or indirectly tied to the early post-application NH_3 spike reported with LA-I. Consistent elevation was also observed for *Pseudactinotalea*, a genus previously linked to poultry litter composting (Chen et al., 2025), and *Bifidobacterium*, a saccharolytic genus negatively associated with *Campylobacter* in poultry (Valeris-Chacin et al., 2021) and widely used in commercial probiotics (Halder et al., 2024).

Prolonged LA-I exposure led to the enrichment of several ecologically relevant taxa. These included the alkaliphilic genera *Alkalibacterium* and *Oceanobacillus*, both identified as broiler litter hub taxa negatively associated with *Campylobacter* (Valeris-Chacin et al., 2022), with *Oceanobacillus* also known to dominate microbial communities during poultry litter composting (Awasthi et al., 2021; Chen et al., 2024). *Facklamia* was similarly enriched; this genus is among the most abundant in poultry litter (Lu et al., 2003) and has been positively associated with peripheral and intestinal mucosal immune indicators in the broiler ileum upon exposure to litter (Song et al., 2022). Additionally, *Facklamia* abundance was elevated in normal ileal feces compared to samples exhibiting feed passage, suggesting a potential link to gut microbial stability (Alvarenga et al., 2023). *Flaviflexus* also showed consistent elevation and has been reported at higher abundance in *Salmonella* Pullorum-negative hens than in diseased counterparts (Niu et al., 2025). *Halomonas* was enriched with prolonged LA-I exposure, consistent with its known increase in biochar-treated poultry litter composts, where it is associated with reduced nitrification and enhanced NH_4^+ retention (Zainudin et al., 2020). It has also been identified as a core member of the broiler litter microbiome (Johnson et al., 2021). Given that *Gottschalkia* was also elevated, co-enrichment with *Halomonas* may reflect cooperation that enhances nitrogen conservation and suppresses nitrifier activity. Such shifts could improve the fertilizer value of reused litter while limiting NH_3 volatilization, even under reduced $NaHSO_4$ input. Additional enrichment patterns, including *Atopostipes* and *Nocardiopsis*, mirrored their presence in the treatment-specific core microbiomes.

Conversely, LA-I treatment was associated with consistent depletion of the halophilic, mesophilic genera *Natribacillus* and *Gracilibacillus*, a pattern potentially linked to reduced $NaHSO_4$ input and a presumable decrease in litter salinity. *Gracilibacillus* has been identified as a key contributor to humic acid synthesis during the mature phase of poultry litter composting (Yang et al., 2024; Bucher et al., 2020), and its decline may indicate altered conditions for late-stage organic matter decomposition. This trend coincided with the enrichment and core microbiome representation of *Nocardiopsis*, which is also associated with humic acid synthesis during the composting maturity phase (Yang et al., 2024). The reduction in *Gracilibacillus* likely increased substrate availability and reduced competitive pressure, facilitating the growth of *Nocardiopsis* under LA-I conditions. This transition may help sustain humic acid production and enhance the fertilizer value of reused litter even under modified salinity and acidification dynamics (Sánchez et al., 2017).

Lastly, *Staphylococcus* was depleted in both LA-I–treated houses compared to the control. This genus, commonly present in poultry litter and on bird skin and mucosa, includes opportunistic pathogens such as *S. aureus* with zoonotic potential (Lowder et al., 2009). Its depletion under LA-I may reflect unfavorable conditions for persistence or competitive exclusion by enriched taxa such as *Actinomycetaceae. In vitro* assessments of minimum inhibitory concentration (MIC) will be necessary to determine whether LA-I directly suppresses *Staphylococcus* or exerts indirect effects via microbial community restructuring.

Implications for litter management and future research

Findings herein demonstrate the potential of microbiome-targeted litter amendments to restructure poultry litter microbial communities in ways that reduce pathogen load and modulate ecological function. Taxonomic shifts were largely consistent across LA-I regimens, suggesting that LA-I establishes a microbial signature early in the flock cycle that persists through to flock removal.

Given that measurements were taken weeks after application, observed compositional differences likely reflect early alterations in microbial succession that influenced downstream community assembly. First, LA-I may have accelerated the thermophilic phase of litter decomposition, advancing the transition to oxidative and maturation stages. This supposition is supported by enrichment of *Halomonas* and *Brevibacterium*, genera associated with nitrogen retention and compost stabilization, as well as *Salinicoccus* and *Atopostipes*, two poultry litter–compost indicators that presumably played a more prominent role in the core microbiomes of LA-I–amended groups. *Bifidobacterium*, including *B. pullorum*, was also elevated in LA-I treatments; while mesophilic, members of this saccharolytic genus produce β-xylanase and preferentially metabolize xylooligosaccharides, components of rice hull hemicellulose, while contributing to volatile fatty acid production (Parajó et al., 2004; Baseer et al., 2023). These shifts suggest that LA-I may promote nitrogen conservation and targeted hemicellulose degradation over prolonged thermophilic breakdown of complex structural biomass.

This accelerated succession may also contribute to the depletion of *Staphylococcus* and *Campylobacter* by flock removal through a combination of elevated thermophilic conditions and sustained microbial dynamics unfavorable to opportunists (Tuomela et al., 2000). Future work incorporating temporal sampling, physicochemical profiles (e.g., pH, moisture, elemental composition), and fungal community dynamics (Manogaran et al., 2022) will be critical for resolving how early interventions influence microbiome compositional changes linked to litter decomposition and poultry health under commercial conditions.

Study limitations and future directions

Several limitations should be considered when interpreting the findings of the current study. First, using composite samples from each house introduced pseudoreplication, limiting the ability to infer within-treatment variability and reducing statistical power. While distinct microbial signatures were observed, overlapping compositional profiles between treatments, particularly

between treated houses, suggest that some observed effects may be subtle or subject to external influences. Additionally, changes attributed to LA-I administration may partly reflect the gradual removal or reduced top-dressing of $NaHSO_4$ over time, as indicated in previous studies demonstrating microbial shifts following the withdrawal of conventional litter acidifiers (Johnson et al., 2021). This complicates the attribution of microbiome changes solely to treatment.

Another important consideration is the absence of performance and physicochemical data (e.g., ammonia concentration, moisture content, bird productivity), which are necessary to contextualize microbiome shifts regarding functional or production-related outcomes. Ammonia-binding products are well-documented for their importance in maintaining poultry welfare and respiratory health (Johnson et al., 2011), and without these benchmarks, it is difficult to evaluate the practical impact of LA-I fully.

Moreover, microbiome composition is susceptible to environmental factors such as litter age, sampling time, and seasonality. These variables were not systematically controlled or analyzed in this study and could influence the generalizability of the findings. Future studies should incorporate replication at the flock or pen level, longitudinal sampling across production cycles, and concurrent measurement of litter chemistry and bird performance metrics. *In vitro* litter models could also help isolate the effects of specific amendment components under controlled conditions. Collectively, these approaches will enable a more mechanistic understanding of how LA-I can shape poultry litter microbiomes and whether those changes confer meaningful improvements to poultry health and environmental outcomes.

Conclusion

The current study demonstrated that integrating a prebiotic litter amendment (LA-I) with reduced $NaHSO_4$ application significantly altered the microbial composition of poultry litter, promoting shifts toward taxa potentially associated with nutrient cycling, organic matter degradation, and reduced pathogen presence. While α diversity remained stable, consistent changes in community structure and differential abundance patterns were observed between treated and control houses. These findings highlight the potential for targeted litter amendments to modulate microbial communities in ways that enhance environmental sustainability and poultry health. Complementing $NaHSO_4$-based acidifiers with IndigoLT™ may offer a synergistic strategy to promote microbiome stability, accelerate litter decomposition, and reduce NH_3 volatilization, thereby improving litter quality and overall flock performance. Future work should include optimizing inclusion rates most complementary between $NaHSO_4$ and IndigoLT™ to maximize efficacy across production systems.

Data availability

The FASTQ files associated with this work have been deposited in the NCBI Sequence Read Archive database under BioProject PRJNA1332267.

Author contributions

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