



Metagenomic insights into the impact of litter from poultry Concentrated Animal Feeding Operations (CAFOs) to adjacent soil and water microbial communities

Ana Durán-Viseras^{a,b}, Blake G. Lindner^b, Janet K. Hatt^b, Amanda Lai^c, Robert Wallace^d, Olivia Ginn^e, Joe Brown^{f,*}, Konstantinos T. Konstantinidis^{b,*}

^a Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, Sevilla 41012, Spain

^b School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA

^c Southern California Coastal Water Research Project, Costa Mesa, CA 92626, USA

^d Georgia Tech Research Institute, Georgia Institute of Technology, Atlanta, GA 30332, USA

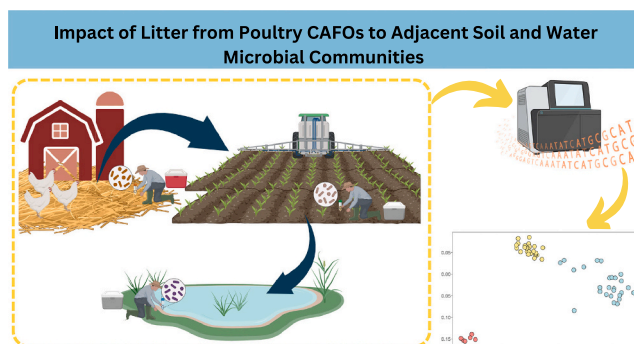
^e Chemical, Materials and Biomedical Engineering Department and Institute for Bioinformatics, University of Georgia, Athens, GA 30601, USA

^f Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

HIGHLIGHTS

- Shotgun metagenomics was employed to investigate the impact of litter application on adjacent soils and downstream creek waters from poultry farms in Southeast US.
- Shifts in the microbial diversity, functional gene content and antibiotic resistance genes (ARGs) in litter-receiving soils were assessed against adjacent control samples (no litter application).
- Litter-associated microorganisms or ARGs were not detectable at the detection limit of our metagenomic sequencing effort in any of the soils or nearby waterways after a couple months of litter application.
- Our findings highlight the high resilience of natural microbial communities to litter perturbations.

GRAPHICAL ABSTRACT



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ABSTRACT

In recent decades, human food consumption has led to an increased demand for animal-based foods, particularly chicken meat production. The state of Georgia, USA is one of the top broiler chicken producers in the United States, where animals are raised in Concentrated Animal Feeding Operations (CAFOs). Without proper management, CAFOs could negatively impact the environment and become a public health risk as a source of water and air pollution and/or by spreading antimicrobial resistance genes. In this study, we used metagenome sequencing to investigate the impact of the application of the CAFO's litter on adjacent soils and downstream

* Corresponding authors.

E-mail addresses: joebrown@unc.edu (J. Brown), kostas@ce.gatech.edu (K.T. Konstantinidis).

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creek waters in terms of microbial diversity and antimicrobial resistance profile changes. Our data indicate that while a few microbial groups increased in abundance within a short period of time after litter application, these populations subsequently decreased to levels similar to those found prior to the litter application or to below the detection limit of our metagenome sequencing effort. Microbial taxonomic composition analyses, relative abundance of Metagenome-Assembled Genomes (MAGs) and detection of Antimicrobial Resistance Genes (ARGs) allow us to conclude that this practice of litter application had a negligible effect on the microbiome or resistome profile of these soils and nearby waterways, likely due to its dilution in the field and/or outcompetition by indigenous microbes, revealing a minimal impact of these poultry facilities on the natural microbial communities.

1. Introduction

Human food consumption has experienced important changes over the past decades, with the demand for foods of animal origin, including milk and dairy products, eggs and meat growing significantly (Henchion et al., 2021). Chicken meat production, in particular, has increased worldwide by over ten-fold since the 1960s (Ritchie et al., 2017). A direct consequence of this growth in poultry consumption is the resulting increase in litter production, defined as the mixture of poultry manure with spilled feed, feathers and bedding materials. Concentrated Animal Feeding Operations (CAFOs) are agricultural facilities where a large number of animals ($n > 1000$) are confined indoors with feeding operations lasting for at least 45 days at a time and no plant crops sustained within the facility (U.S. Environmental Protection Agency, 2001). CAFOs raising chickens are prevalent throughout the United States, especially in the Southern states (Georgia, Alabama, Arkansas, North Carolina and Mississippi), and are the major source of poultry meat sold at markets across the country (National Chicken Council, 2021). According to the United States Department of Agriculture (USDA), the number of broilers chickens produced in the state of Georgia alone reached the number of ~1.3 billion heads in 2021, representing ~14 % of the total broiler chicken production in the United States (National Agricultural Statistics Service, 2022).

CAFOs produce large quantities of animal waste within very small areas which can contribute to problems of water and air pollution (e.g., odorous gases such as ammonia or uncontrolled greenhouse gas emissions). These issues of CAFO driven environmental pollution are exacerbated by variable waste management practices such as, for example, the direct application of waste to agricultural land as fertilizer (Bolan et al., 2010; Mallin and McIver, 2018). Thus, several environmental and human health risks are frequently associated with CAFO facilities (Ayala-Ramirez et al., 2023). Other potential threats to public health include the widely extended practice of antibiotic use in animal agriculture for prophylaxis, therapy, and growth promotion (Van Boeckel et al., 2015). There are growing concerns about antimicrobial resistance (AMR) development within CAFOs and its spread into the nearby environment and associated bacterial communities (Hu et al., 2016; Wang et al., 2012). Studies have shown that AMR genes may enter the environment through runoff, leaching, or application of animal waste as manure to land, all of particular concern in poultry operations (Chee-Sanford et al., 2009; Martinez, 2009; Zhang et al., 2009). Nevertheless, due to the yet-limited number of available studies, there is a need to understand and quantify to what extent the microbiota from the animal wastes and the antimicrobial resistance genes (ARGs) are transferred to the adjacent natural microbial communities and downstream waters as a consequence of different waste management practices. The limited number of studies available to date have mainly focused on the impact of manure application on land through 16S rRNA gene (or simply 16S) amplicon sequencing or quantitative PCR approaches (Liu et al., 2020; Riber et al., 2014; Xie et al., 2018). However, very few studies have focused specifically on litter, investigated the impact of animal waste in nearby waterways, or used metagenomic techniques. Moreover, in situ studies are rare, with several relying on ex-situ experiments (Han et al., 2018; Wang et al., 2017; Zhang et al., 2017).

The purpose of this study was to evaluate, using a culture-independent metagenomic approach, the effect of several CAFOs facilities on adjacent natural soil and water microbial communities, including in their resistome, by following the microbial community composition in these systems over time after receiving the litter application. We also investigated whether or not the microbial communities recovered to the pre-application state in terms of community ecology and ARG prevalence. To the best of our knowledge, this is one of the first studies to examine the impact of CAFO litter on the adjacent soil and water microbial communities in-situ via metagenomic techniques following application of chicken litter to land.

2. Materials and methods

2.1. Sample collection

Six litter samples were collected across six CAFO sites at 1–2 sampling events per site (Table S1). Litter sample collection days occurred within 24 h after birds were vacated from the house. For each sample, 10 litter samples were collected along the length of drinker lines inside grow houses and treated as one composite sample. Sampling dates were determined by growers and dependent on flock grow out dates. In total, 21 land-applied soil samples receiving litter from the CAFO were taken across six CAFOs at two sampling events per location (Table S1). Litter was applied to the sampled land by the growers using a “manure spreader”. Three soil samples were taken at each sampling event at a depth of 0–15 cm and on a 10 × 10 cm grid and treated as individual samples (not a composite). Litter handling practices varied between individual farms, making it difficult to control for the imprecise and varied methodologies used in situ. For example, most operations stored spent litter in sheds for different amounts of time before land application, making it difficult to determine the exact age of litter actively being land applied. Nonetheless, when possible, soil samples were collected at the uphill, mid-hill, and downhill boundaries of the land-applied areas feeding into a receiving stream. The time between sample collection and, the most recent land application event varied between 21 days and 365 day with an average of 109 days. Access to facilities and land and the schedule of growers performing land application were not such that regular intervals of sampling could be obtained. Soil samples with no history of litter application were also collected and used as negative controls. In addition to soil samples, 21 water samples were taken in receiving waters downstream of six CAFOs at two sampling events per location where land-applied litter and contaminants may have been mobilized via runoff or subsurface transfer into receiving waters (Table S1). Three samples (50-mL/each) were collected at each sampling event using hollow-filter dead-end ultrafiltration (DEUF) for recovery of enteric pathogens (Mull and Hill, 2012). When possible, samples were collected at the point of discharge, ~1 km downstream of the point of discharge, and 1–5 km further downstream. Water samples were also collected downstream from the soil control sites and used as controls. The most upstream samples were located between 0.5 km and 1 km from land-applied soil sampling sites. Receiving waters were streams located on or directly adjacent to the farm property that fed into a river.

2.2. DNA extraction

DNA extraction was performed on litter and soil samples with 0.2–0.5 g of litter or 10 g of soil, using Qiagen's PowerFecal kit and Qiagen's PowerMax Soil kit, respectively. For the water samples, DNA extraction was performed with 75–750 µL of resuspended PEG pellet using Qiagen's PowerFecal kit, following the manufacturer's protocol with a modified 30-second bead-beating lysis step. DNA concentration was measured using a Qubit™ 3.0 Fluorometer and purity was determined by measuring the absorbance ratios at 260/280 and 260/230 using a Nanodrop 1000 Spectrophotometer.

2.3. Metagenomic sequencing

The DNA samples were prepared for sequencing by using the Nextera XT DNA library prep kit manufactured by Illumina, San Diego, CA. The resulting DNA libraries were sequenced using an Illumina HiSeq 2500 instrument available at the Georgia Institute of Technology High Throughput DNA Sequencing Core. Adapter trimming and demultiplexing of the samples were carried out on the sequencing instrument.

2.4. Sequence data analysis

Raw data were quality assessed and trimmed using FaQCs 2.10 (Lo and Chain, 2014). Reads with a quality lower than 15 and shorter than 50 bp after trimming were removed. The Nonpareil tool 3.401 (Rodríguez-R and Konstantinidis, 2014) with default parameters was used to estimate the microbial community coverage by each metagenomic dataset. Overall similarities between metagenomic datasets were determined based on Mash distances (Ondov et al., 2016) for the trimmed reads and visualized using a PCoA plot generated with the ggplot2 package in R (Wickham, 2011). Permutational multivariate analysis of variance using distance matrices was performed with the adonis function of the R package vegan (Dixon, 2003).

Kraken2 was used to assign taxonomy (at the rank of class, order, family and genus) against a reference library, including bacteria, archaea, viruses, protozoa, human, and fungal reference genomes (Wood et al., 2019). The relative abundance of the Kraken2 profiles was estimated by Bracken as performed previously (Lu et al., 2017).

Quality-trimmed reads were assembled independently, de novo with Spades 3.15.4 (“-meta”) (Prjibelski et al., 2020) and IDBA-UD 1.1.3 (Peng et al., 2012) and the generated assemblies were quality checked using metaQuast (Mikheenko et al., 2016). Contigs shorter than 5 Kbp from both assemblies were removed prior to population genome binning, which was performed independently by MaxBin 2.2.7 and MetaBAT 2.12.1 with default parameters (Kang et al., 2019; Wu et al., 2016). All resulting Metagenome-Assembled Genomes (MAGs) from the same sample and the different assembly and binning runs were dereplicated using dRep 3.4.0 (Olm et al., 2017) at ANI 95 %. MAG contamination and completeness was assessed with CheckM 1.1.2 (Parks et al., 2015). MAG quality was calculated as “Quality = Completeness – (5 × Contamination)”, and only medium-to-high-quality MAGs (quality score ≥ 50) were used for further analysis. To assign the taxonomy to the generated MAGs the toolkits GTDB-Tk v2.2.6 and GTDB r207_v2 (Chaumeil et al., 2020), and Microbial Genome Atlas (MiGA) v1.3.8.3 and TypeMat databases (Rodríguez-R et al., 2018) were used. This process resulted in 31 medium-to-high-quality MAGs (Table S3) for the litter samples that represented the genome diversity recovered in each sample. Dereplication across litter samples using the same 95 % ANI cut-off resulted in 13 MAGs representing the species-level diversity recovered in the samples.

The abundance and presence of recovered MAGs was assessed by read recruitment. For this, individual MAG sequences were mapped against all metagenomic reads from each dataset using stand-alone BLASTn (best match when better than 95 % nucleotide identity and 70 % of read length were used to identify mapped reads). Read

recruitment plots were obtained using the enveomics collection (Rodríguez-R and Konstantinidis, 2016) and visually inspected for presence/absence. Alternatively, the 80 % truncated average sequencing depth (TAD80) of each MAG in each sample was estimated using coverM 0.4.0 (<https://github.com/wwood/CoverM>) with the following arguments: `coverm genome -p bwa-mem -min-read-percent-identity 95 -min-read-aligned-percent 75 -trim-max 90 -trim-min 10 -m trimmed_mean`. TAD80 values were further normalized by the genome equivalent (GEQ) of each metagenome using MicrobeCensus (Nayfach and Pollard, 2015), which represents a more accurate metric of abundances that accounts for average genome size differences among metagenomes. The normalized abundances were represented by heatmap using the ggplot2 package in R (Wickham, 2011). To predict the estimated theoretical limit of detection (thLOD) of the microbial populations, the methodology previously described by Castro et al., 2018 and Lindner et al., 2022 was followed under the assumption of 1×10^8 cells per gram and 1×10^6 cells per milliliter for soil and water samples, respectively (Grenni et al., 2014; Lopes et al., 2011). That is, the target populations must be present in an abundance higher than the estimated thLOD in order to be detected by our sequencing effort.

The functional diversity of the metagenomes under study was assessed as follows: genes were predicted on assembled contigs using Prodigal v2.6.3 (Hyatt et al., 2010), followed by gene clustering using MMseqs2 v13.45111 (Steinegger and Söding, 2017) at 90 % amino acid sequence identity. The resulting gene clusters were subsequently annotated using the eggNOG-mapper v2.1.12 and the eggNOG 5.0.2 database (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019). The gene diversity was assessed based on the fraction of total genes assigned to each functional category.

2.5. Detection and quantification of antimicrobial resistance genes (ARGs)

The detection of ARGs was performed using the tool ResFinder (Florensa et al., 2022) based on the metagenomic reads BLASTn mapping against the “Acquired antimicrobial resistance genes” database, with the following arguments: minimum identity 90 % and minimum read coverage length 60 %. The relative abundance of each gene was calculated by normalizing the average sequencing depth of each gene to the GEQ estimate of each metagenome. Results were represented by bubble charts using the ggplot2 package in R (Wickham, 2011).

Alternatively, to estimate the abundance of β -lactamase and tetracycline encoding genes in the metagenomic datasets under study, short reads were mapped against the reference gene sequences of precompiled 150 bp β -lactamase and tetracycline ROCKER models (Suttner et al., 2020; Zhang et al., 2022) by using the software DIAMOND 2.0.1 (blastx –ultra-sensitive) (Buchfink et al., 2015). Mapped reads were selected for best bit-score alignment and subsequently filtered by ROCKER v1.5.2 as described previously (Orellana et al., 2016). The relative abundance of each ARG class was calculated by normalizing the average sequencing depth of each gene to the GEQ of the corresponding metagenome and summing across each class. Results were visualized as a heatmap using the ggplot2 package in R (Wickham, 2011).

3. Results

3.1. Description of sampling locations

Six different CAFOs raising broiler chickens located in the north of the state of Georgia were sampled in this study. A total of 58 samples were collected: six from the deep litter from the different CAFOs, 21 from soils receiving land application of spent litter, and 21 from water sources located downhill of the CAFO or land application site (Table S1). At each CAFO, soil and water samples were taken at two different time points after litter application on land, hereafter designated as visit 1 (T1) and visit 2 (T2) (Table S1). Dates elapsed between the litter application

on land and sampling ranged from 21 days up to 365 days (Table S1). Additionally, for comparison, 12 control samples were also collected (Table S1). These samples (six from soils and six from water) represented six different locations with downhill water sources where no litter was applied on soils. Note that while six (different) sites were sampled for litter (sites 1, 3, 4, 5, 7, and 8), companion soil and water samples were collected for only three of the sites (1, 3, and 5) and one additional fourth site (9), which was not sampled for litter, due to restricted access during field work (see Table S1 for details).

3.2. Bacterial community structure in CAFO samples and effects of litter application at the whole-community level

A total of 58 metagenomes were obtained from the six CAFOs, ranging in size between 6.89 and 33.9 million Illumina short-read sequences (0.89 to 5.1 Gbp) after trimming (Table S2). For these samples, an average of 84.7 %, 17 % and 24.3 % of the total community for litter, soil, and water, respectively, was recovered by our sequencing effort based on Nonpareil analysis (Table S2). Of note, to be able to recover 95 % of the total community in soil and water, ~250 Gbp and ~150 Gbp of short-read sequencing would be needed, respectively. Consequently, the assembly of the litter metagenomes was more successful than those from soil and water, which is also consistent with the higher diversity expected in the latter two habitats. Consistently, Nonpareil sequence diversity (N_d) values showed that the soil and water samples were more diverse than the litter samples (Fig. 1A). On the other hand, the level of diversity of the soil and water samples as well as diversity patterns over time (T1 and T2) did not differ from those of the soil and water control samples, respectively (Fig. 1A).

Observations of beta diversity among samples revealed a clear

clustering of samples based on their nature (litter, water or soil; p -value: 0.001) (Fig. 1B). Similarly, no significant differences were observed in terms of beta-diversity at time-points (T1 and T2; p -value: 0.872) after the litter application on soils (Fig. 1C). Notably, water samples showed a clear clustering pattern based on the sampling site (p -value: 0.001), a pattern that was not observed for soil sites (Fig. 1C and D).

To explore the microbial diversity of the different CAFO samples at the individual taxon level, the community composition of the generated datasets was characterized using a k-mer based approach as implemented in the Kraken2 software followed by Bracken. In soil communities, *Streptomycetaceae* (5.8–8.4 %), *Nitrobacteraceae* (4.6–11.3 %), *Comamonadaceae* (3.6–6.6 %), *Burkholderiaceae* (3.5–5.6 %) and *Sphingomonadaceae* (2.9–5.9 %) were the most abundant families, whereas in litter communities, and consistent with previous literature (Cressman et al., 2010; De Cesare et al., 2019; Wang et al., 2016), *Brevibacteraceae* (5.6–38.36 %), *Corynebacteriaceae* (8.3–37.7 %), *Dermabacteraceae* (7.6–15.5 %), *Staphylococcaceae* (3.5–15.7 %) and *Lactobacillaceae* (1.4–6.5 %) were dominant. In water, the most abundant bacterial communities at the family level were *Comamonadaceae* (4.1–40.2 %), *Pseudomonadaceae* (2.0–16.5 %) and *Burkholderiaceae* (3.2–10.2 %) (Fig. 2). No differences were observed between the different time points.

3.3. Tracing litter-derived microorganisms in soils

The presence and differential abundance analysis over the time of specific litter-associated taxa over time was examined in more detail, based on the Kraken and Bracken results, to assess their persistence in the environment and detect any finer impacts of litter application. The average relative abundance of the 10 most abundant genera in the litter samples showed that they were practically absent in the soil samples

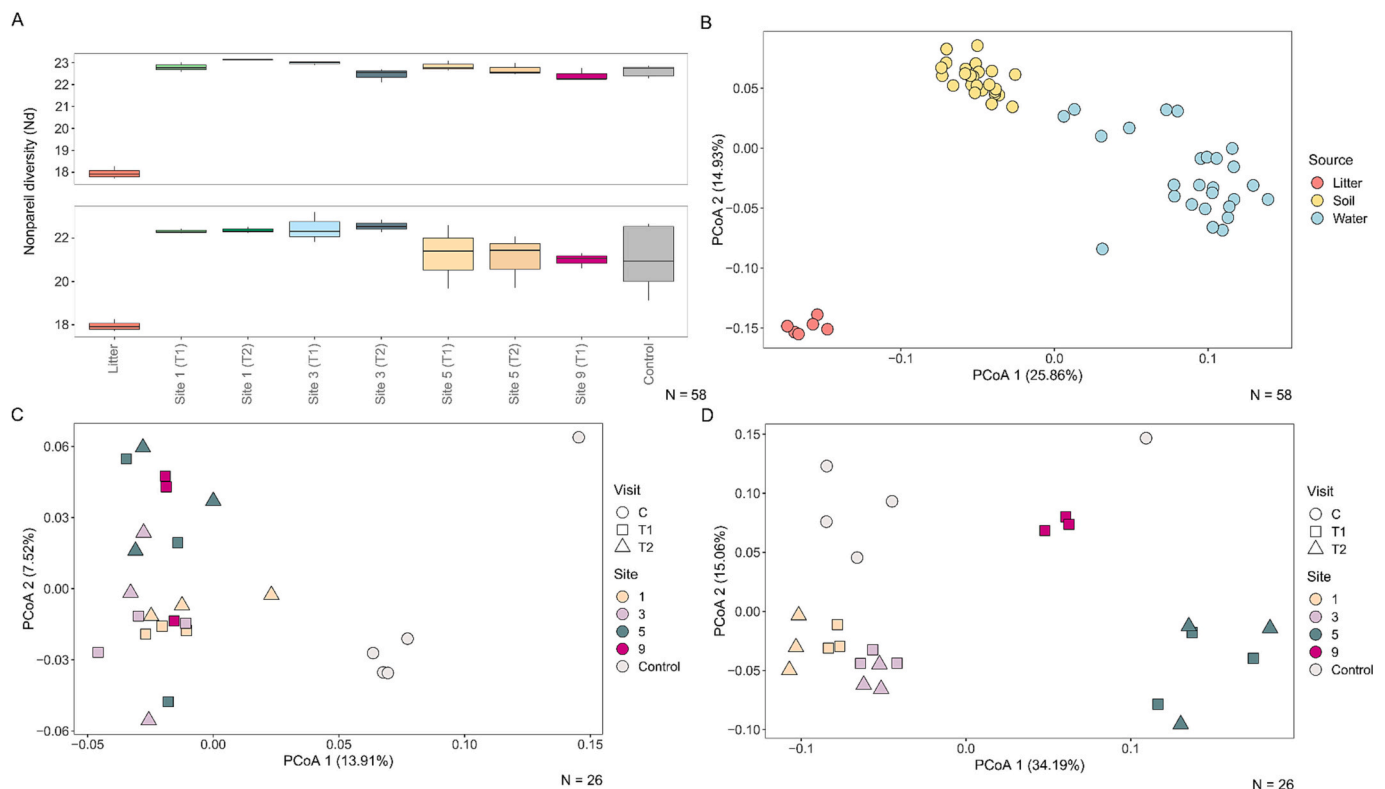


Fig. 1. Nonpareil diversity (N_d) and mash distance comparison of the samples used in this study. (A) N_d values for litter and soil samples (upper plot) and litter and water samples (lower plot) are shown. The error bars represent the standard deviation within each group of samples. (B) Principal coordinate analysis (PCoA) based on mash distances illustrating the clustering of samples by source (litter, soil, water), (C) by CAFOs and sampling time-point for soil, and (D) water samples. Sites refer to the different CAFOs where the samples were collected, T1 indicates visit 1, T2 indicates visit 2, and C indicates control. Days elapsed between the different visits and litter application on land for each site were: 88 days (T1) and 162 days (T2) (Site 1); 35 days (T1) and 56 days (T2) (Site 3); 365 days (T1) and 21 days (T2) (Site 5); 41 days (T1) (Site 9).

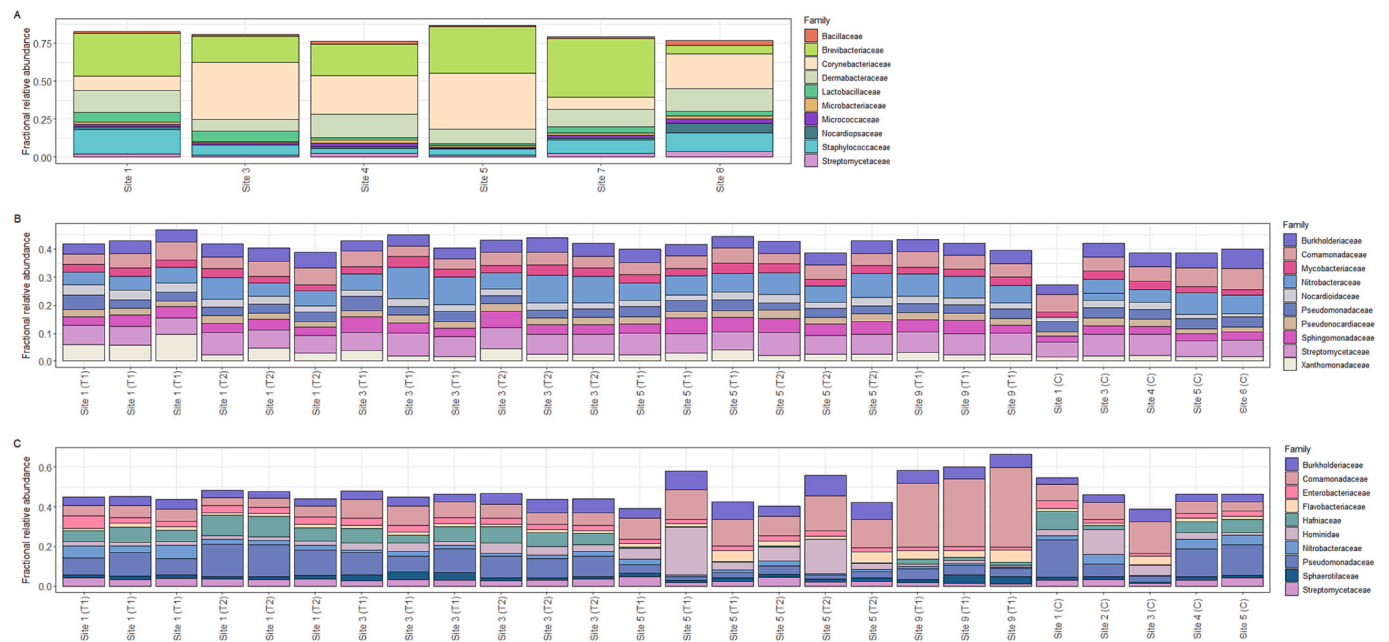


Fig. 2. Relative abundance of the top ten most abundant bacterial families in litter (A), soil (B) and water (C) metagenomic samples. The underlying data are metagenomic short read annotated by Kraken/Bracken. The X axis depicts the different CAFOs (sites) where the samples were collected, T1 indicates visit 1, T2 indicates visit 2, and C indicates control. Days elapsed between the different visits and litter application on land for each site were: 88 days (T1) and 162 days (T2) (Site 1); 35 days (T1) and 56 days (T2) (Site 3); 365 days (T1) and 21 days (T2) (Site 5); 41 days (T1) (Site 9).

with relative abundances ranging from 0.001 % up to 0.8 % (Fig. 3A), with the sole exception of the genus *Streptomyces*, a well-known group of soil microorganisms. Indeed, the average relative abundance of *Streptomyces* in litter was 2 % contrasting with an average of 6.8 % and 6.2 % in land-applied soils and controls, respectively. Similar patterns were observed in the water samples (Fig. 3B).

Notably, certain litter-associated taxa such as *Staphylococcus* and *Nocardopsis* (average relative abundances in litter of 5.7 % and 1.7 %, respectively) were detectable in samples taken shortly after litter

application, e.g. 21 days after land application (Site 5), with relative abundances up to 0.7 % and 0.2 %, respectively; decreasing to values around 0.12 % and 0.17 % respectively, at the following time-point, e.g. 35 days (Site 3) (Fig. 3A). In contrast, soil controls exhibited average values of 0.11 % and 0.14 %, for *Staphylococcus* and *Nocardopsis*, respectively. Thus, these data indicated that the indigenous microbial community is indeed affected by the litter application in the short term, but it is able to recover to the pre-application state after about a couple months.

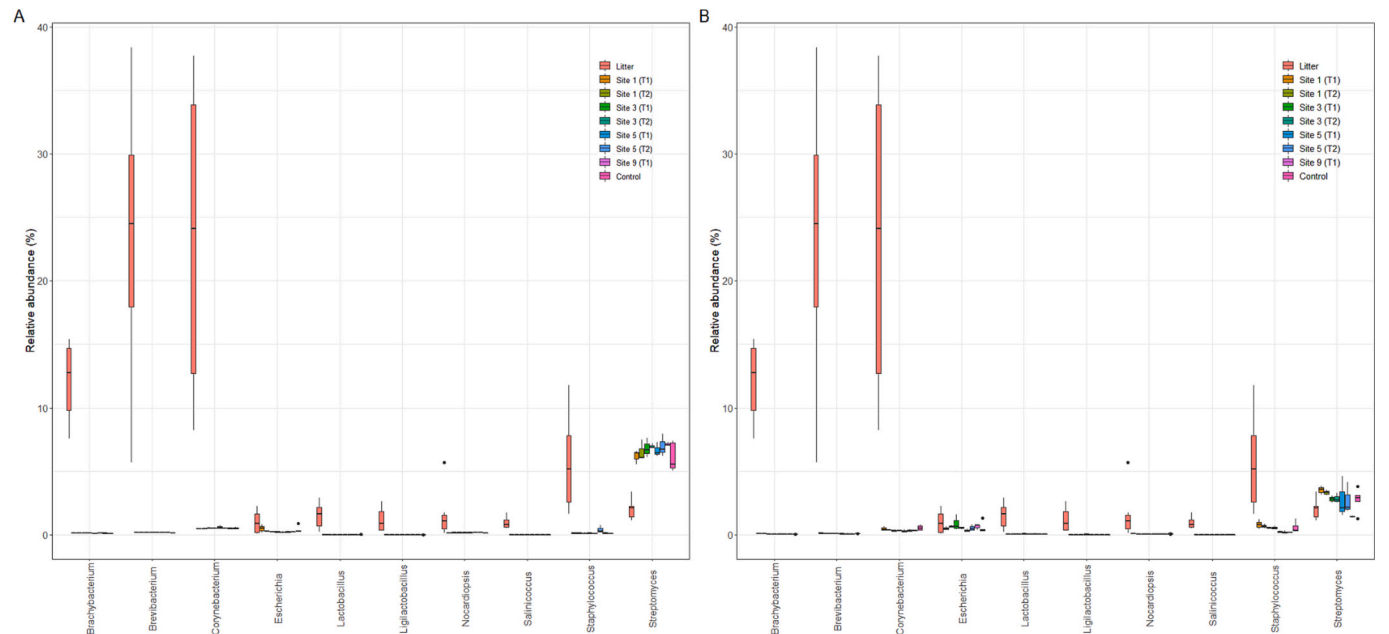


Fig. 3. Average relative abundance of the top ten most abundant genera from litter (according to Kraken) in litter and soil samples (A), and in litter and water samples (B). The underlying data are metagenomic short read annotated by Kraken/Bracken. The colors in the legend depict the different CAFOs (sites) where the samples were collected, T1 indicates visit 1, T2 indicates visit 2, and C indicates control. Days elapsed between the different visits and litter application on land for each site were: 88 days (T1) and 162 days (T2) (Site 1); 35 days (T1) and 56 days (T2) (Site 3); 365 days (T1) and 21 days (T2) (Site 5); 41 days (T1) (Site 9).

Likewise, the overall relative abundance of groups such as *Escherichia* or *Corynebacterium* (1 % and 23.4 % average relative abundances in litter, respectively) was higher in soils (with relative abundances on average of 0.3 % and 0.5 %, respectively) compared to the other litter-associated taxa analyzed (average abundances for those groups ranging from 0.001 to 0.18 %) (Fig. 3A), suggesting that these genera might be more resistant to soil conditions, and thus persist in the environment for a longer period of time. However, these two genera exhibited similar relative abundances in both amended and control soils, suggesting that their native populations in these soils might be responsible for the results obtained, especially in later sampling time points, rather than the effect of the litter application. For the remaining five genera, no changes were observed along the different time-points, remaining non-detectable in all environmental samples. Nevertheless, for more accurate estimations about the litter impact in soils we investigated at the MAGs level.

3.4. Metagenome-assembled genomes (MAGs) of litter

A total of 334 MAGs were recovered from binning of the six litter sample assemblies (samples were binned individually), resulting in a selection of 13 medium-to-high-quality (completeness – [5*contamination] ≥ 50) MAGs after dereplication across the litter samples at the ANI ≥ 95 % level (31 MAGs before dereplication across samples). The genome size of the selected MAGs ranged from 1.3 to 4.1 Mbp, while their G + C% content ranged from 33.0 to 71.8 mol% (Table S3). Comparison of the selected MAGs against the GTDB-tk database revealed matches to previously described taxa at the species level (>95 % ANI) for the majority of MAGs, while one and five MAGs potentially represented a novel genus of a matching family (*Jiangellaceae*), and a novel species of a matching genus (*Brachybacterium*, *Corynebacterium*, *Nocardiopsis*, and *Oceanisphaera*), respectively (Table S3). These results were further confirmed by searching against MiGA's TypeMat database (Rodríguez-R et al., 2018), and the only differences observed were the species names recently proposed by Gilroy et al. (2021) that have not been validated yet by the International Code of Nomenclature of Prokaryotes (Table S3). The MAGs were assignable to three different phyla, *Actinobacteriota*, *Firmicutes* and *Proteobacteria*, which are commonly found in the chicken gut microbiome as the predominant phyla (Glen-dinning et al., 2020; Segura-Wang et al., 2021; Xiao et al., 2017; Yang et al., 2022). These findings are also consistent with the microbiota of chickens from other parts of the globe such as Europe and China (Feng et al., 2021; Yang et al., 2022), indicating that the results reported here on the chicken commensal gut microbiota may apply more broadly, at this (the phylum) level.

The most dominant phylum was *Actinomycetota* (comprising 61.3 % of the total 31 MAGs obtained, $n = 19$) with genome representatives from five different genera (*Brevibacterium*, *Brachybacterium*, *Yaniella*, *Corynebacterium*, and *Nocardiopsis*), followed by *Bacillota* (35.5 % of the total MAGs, $n = 11$) with genome representatives from four different genera (*Atopostipes*, *Jeotgalicoccus*, *Salinicoccus*, and *Staphylococcus*) (Table S3). For the phylum *Pseudomonadota* there was only one MAG belonging to the genus *Oceanisphaera* (Table S3). Indeed, most of these genera correspond to the most abundant groups identified by Kraken2 on unassembled metagenome reads of our litter samples, as shown above.

3.5. Fate of litter MAGs in soils and water

To further corroborate the whole-community results reported above and obtain finer resolution regarding the fate of litter-associated populations in the natural environment, the relative abundance of the recovered litter MAGs was estimated using read recruitment, followed by normalization of the resulting relative abundance by the TAD80 metric (to avoid spurious matches) and GEQ (to provide a normalized estimate of abundance in terms of fraction of total detected genomes) for more accurate estimates. Competitive read mapping of the litter reads

against a total of 13 recovered medium-to-high-quality MAGs resulted in 49.4 % reads recruited per litter metagenome, on average, supporting that our collection of MAGs robustly represents a high percentage of the litter diversity.

Litter-associated MAGs were absent, at the limit of our sequencing effort, in all soil and water datasets (Fig. 4), and thus were exclusively present in the litter metagenomes with a relative individual abundance in the litter metagenomes ranging from 0.0043 % to 7 % of the total (Fig. 4). All litter assembled contigs were also searched against the soil and water metagenomic reads in case any litter-associated members not represented by our MAGs were detectable in these samples. Similar results to those observed with the MAGs were obtained, i.e., no litter contigs were detectable in soil or water (data not shown). Consistently, no overlapping contigs between litter and soil metagenomes that had >99 % identity and >1 Kbp long overlap were detectable, whereas litter metagenomes shared about ~ 50 % of their total contigs in comparison. The average estimated theoretical limit of detection (thLOD) in our metagenomic datasets was estimated to be $2.42\text{E}+04 \pm 5.75\text{E}+03$ (cells/g) and $4.28\text{E}+02 \pm 1.35\text{E}+02$ (cells/mL) in soil and water, respectively (for key assumptions, see the Materials and methods section). Therefore, our target MAG populations from litter, if present in the sampled soils or waters, should be at abundances lower than $2\text{E}+04$ cells/g and $4\text{E}+02$ cells/mL, respectively.

On the other hand, the most abundant MAGs in the litter samples were *Brevibacterium intestinaevium*, *Atopostipes pullistercoris*, *Brachybacterium merdarium*, and *Yaniella excrementigallinarum* being especially prevalent at the CAFOs from the Sites 1, 5 and 7; Site 3; Sites 1, 4 and 8; and Sites 1, 4, 7 and 8; respectively (Fig. 4). Interestingly, these four species have been recently proposed as novel members of the chicken gut microbiome based on a culture-independent study (Gilroy et al., 2021), but studies reporting on their prevalence in the chicken gut microbiome and litter have been limited to date.

3.6. Metabolic pathway abundance and diversity in soils after litter application

We also examined if the application of litter had any effects in terms of broad functional gene content on the soils that received it by comparing the relative abundance of major metabolic pathways related to carbon and nitrogen cycling and energy generation between these soils and their controls. We found limited differences overall between the two groups, e.g., most metabolic pathways showed similar abundances between the two groups. A few significant differences were noted such as a higher abundance of methanogenesis genes in the litter-applied soils vs. controls (e.g., 14 % vs. 11 % of the total genes annotated; Fig. S1), which was consistent with the addition of extra carbon to the soil as an effect of the litter application. Overall, however, the effects of litter on metabolic pathways were rather limited, consistent with our results reported above showing that litter-associated genes and genomes did not survive more than a couple months in the soil.

3.7. Antibiotic resistance gene (ARG) abundance in litter and fate in adjacent soil and water

Reads were screened for the presence of ARGs to assess the fate of these genes due to their relevance for public health. In total, genes coding for 46 subtypes, belonging to 9 different ARG types or families, were found, according to Resfinder (nucleotide level), in at least one of the six litter samples studied, with relative abundances ranging from 0.0016 to 0.4 copies/GEQ (Table S4). None of these ARGs were found in any of the soil samples using the same criteria for presence, while only a couple of ARGs (*blaACC-3*, *lucC*, *tetA*, *tetQ*), with relatively low abundances (0.005 to 0.15 copies/GEQ), were identifiable in a few of the water samples, including controls (Table S4). The latter ARGs were absent in any of the litter samples, suggesting that their presence in water was likely independent of the litter application on adjacent soils.

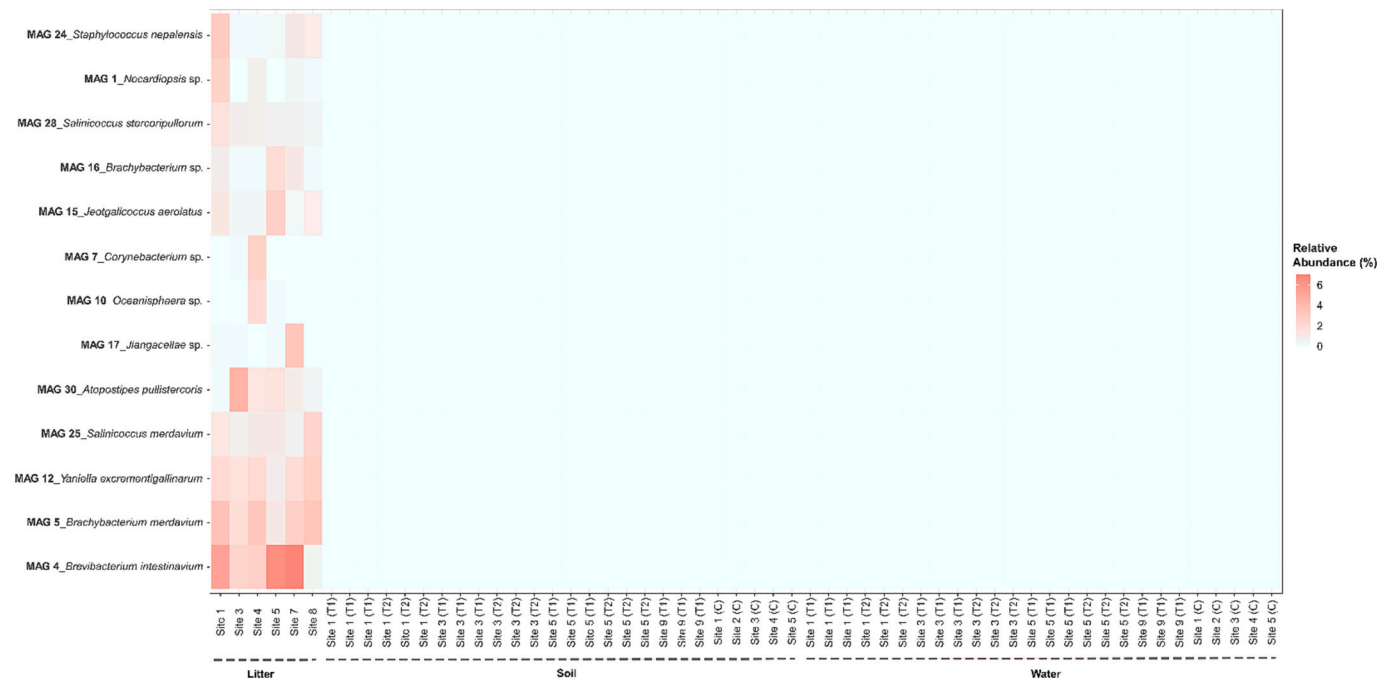


Fig. 4. Relative abundance of the litter MAGs in the different metagenomic datasets from litter, soil and water. Abundance was estimated based on the TAD80 metric normalized by GEQ and represented as a heatmap. Relative abundance values were multiplied by 100 to be expressed in percentages as shown in the legend. The X axis depicts the different CAFOs (sites) where the samples from litter, soil and water were collected, T1 indicates visit 1, T2 indicates visit 2, and C indicates control. Days elapsed between the different visits and litter application on land for each site were: 88 days (T1) and 162 days (T2) (Site 1); 35 days (T1) and 56 days (T2) (Site 3); 365 days (T1) and 21 days (T2) (Site 5); 41 days (T1) (Site 9).

The genes detected in the litter samples encoded resistance to tetracyclines, macrolides, aminoglycosides, sulphonamides, trimethoprim, fosfomycin, lincosamide and phenicol. Overall, resistance genes to trimethoprim, sulphonamides, macrolides and tetracyclines were the most abundant (Fig. 5), with the top five abundant ARG subtypes being *ermC*, *dfrD*, *tetK*, *tetL* and *sul1* (Fig. S2), consistent with previous studies

of the chicken gut resistome (Feng et al., 2021; Qu et al., 2008; Yang et al., 2022). In terms of ARG type diversity, the aminoglycosides, tetracyclines and macrolides were the most diverse groups considering the number of different subtype genes recovered for each of these families (Fig. 5). The most prevalent ARG subtypes that were commonly found in all analyzed litter samples were *aadA9*, *dfrD*, *erm36*, *ermC*, *ermX*, *lnuA*,

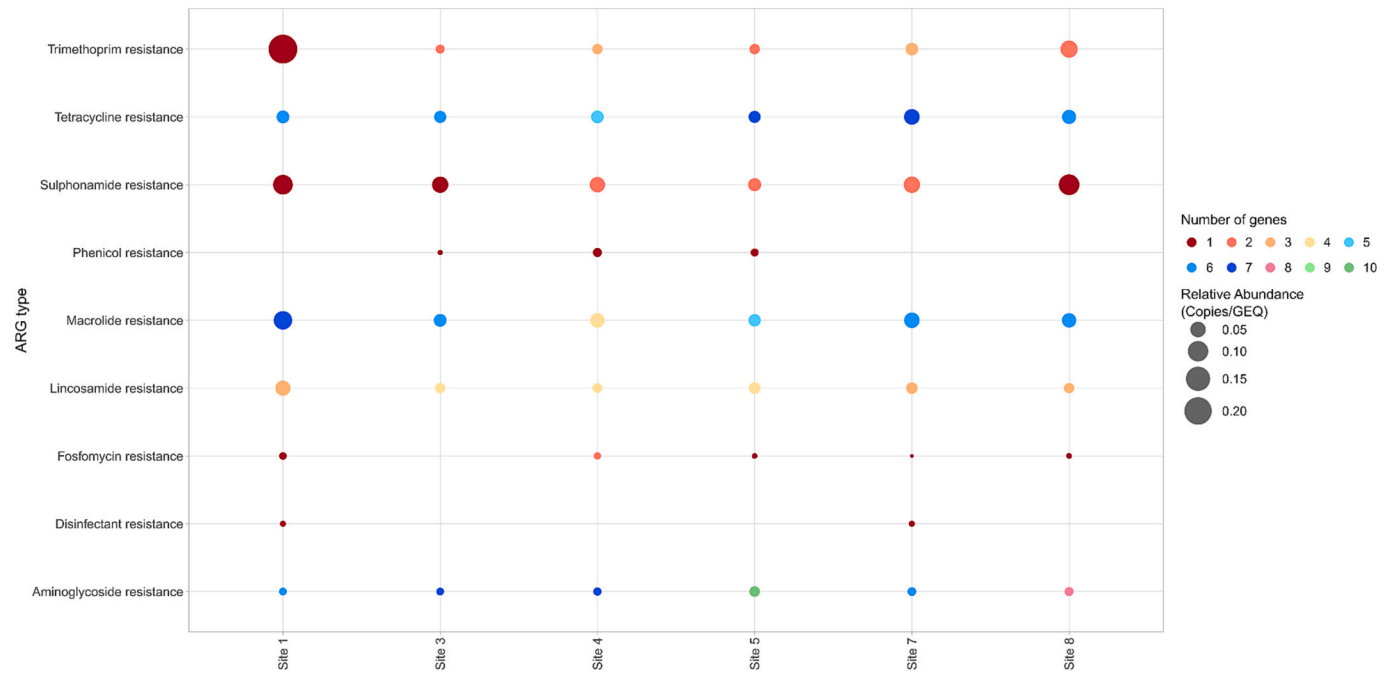


Fig. 5. Relative abundance of each ARG family and the number of ARG subtypes found under each ARG type category in the litter metagenomic samples. The abundance of each ARG type or family represents the sum of the relative abundances (copies/GEQ) of each ARG subtype gene, normalized by the number of genes under that type. The X axis depicts the different CAFOs (sites) where the samples were collected.

hluG, *sul1*, *tet33*, *tetL*, *tetM* and *tetZ* (Fig. S2).

Since genes coding for tetracycline resistance were among the most abundant and frequent ARG subtypes found in the litter samples, we wanted to investigate their distribution in our samples in more detail. For this purpose, we used the previously built ROcker models for tetracycline (*tetM*) and beta-lactamase (*blaA*, *blaC*, MBL and OXA) class genes, an accurate approach to find and type metagenomic reads containing a target gene of interest that avoids spurious matches. With respect to the tetracycline *tetM* gene, and consistent with the ARGs results mentioned above (Table S4), litter samples had the highest relative abundance of this gene (up to 0.13 copies/GEQ) (Fig. S3). The relative abundance of *tetM* in soil and water samples was notably lower than in litter (ranging from 0.007 to 0.07 copies/GEQ), and exhibited similar relative abundance in the soil and water control samples (from 0.004 to 0.05 copies/GEQ) (Fig. S3), suggesting that its presence in the environment might be independent of litter application and most likely part of the natural soil resistome. On the contrary, the relative abundance of beta-lactamase genes was higher in soil and water metagenomes (ranging from 0.005 up to 0.2 copies/GEQ, and 0.0008 up to 0.07 copies/GEQ; respectively) than in litter metagenomes (from 0.0002 to 0.03 copies/GEQ) (Fig. S3). The similar relative abundances values in soil and water samples with respect to their controls (relative abundances ranging from 0.003 to 0.17 copies/GEQ, and 0.004 to 0.08 copies/GEQ; respectively) further supporting their source being independent from litter. The beta-lactamase gene with the highest relative abundance was the MBL class, especially in soil, while the OXA class, *blaA* and *blaC* genes showed similar abundances within the samples (Fig. S3). The fact that ROcker uses a more sensitive approach and might detect more distant homologs likely explains why those genes were not detected by Resfinder.

4. Discussion

Our study examined the changes to the soil and downstream water microbial community composition and antibiotic resistance profiles as an effect of litter application at six CAFOs in Georgia (USA) based on metagenomic sequencing. The chicken gut microbiome is a well-appreciated reservoir of ARGs, and the practice of direct application of the chicken litter to adjacent agricultural lands might be associated with potential environmental and human health threats from spreading ARGs and microbial pathogens. Our data based on a robust abundance estimation metric (TAD80/GEQ) revealed that none of the litter-associated MAG representatives were detected, at the theoretical limit of detection (thLOD) of our sequencing effort, in any of the soil or water datasets a month or longer after litter application. Considering that our collection of MAGs represented well the microbial litter community, these findings indicate that litter application on soils may not introduce persistent microbial populations, possibly due to dilution in the field and/or that the litter-associated taxa do not sustain large populations in the extra-intestinal environment for more than a few days. This hypothesis is consistent with previous studies suggesting that litter-derived bacteria are not well adapted to survive in the extra-intestinal environment (Jechalke et al., 2014). Similarly, no significant changes were observed on the N_d and beta diversity in soil and water samples across the different time-points, and with respect to the controls, further corroborating that the overall microbial composition is not substantially affected by the litter application on soils. These results are consistent with similar studies in which animal manure was applied to soils (Macedo et al., 2021; Poulsen et al., 2013; Riber et al., 2014), suggesting that the higher microbial diversity observed in soils compared to litter and/or the robustness of the indigenous soil microbial community against amendments with exogenous microbes are likely responsible for our findings. Note, however, that litter-associated taxa could survive in the extra-intestinal environment as part of the rare biosphere or resting cells (Gonzales-Siles and Sjöling, 2016; Kreling et al., 2020; Lynch and Neufeld, 2015), which our study's methods could not assess.

Moreover, our data from the metagenomic short read annotation by Kraken/Bracken suggests that, after a short period of time (e.g., less than one month), the relative abundance of specific litter-associated taxa in soils may have increased slightly but, after a couple months, the microbial communities recovered to the composition prior to litter application. This result indicates that variations in the soil microbiome may be transient and microbial communities are resilient, as also has been previously observed by other studies (Lopatto et al., 2019; Rieke et al., 2018). Moreover, additional analyses and sampling are needed to confirm that the increase in the abundance of litter-associated taxa observed (Kraken/Bracken results) is simply not attributable to the growth of close relatives that are indigenous in soils and apparently favored by litter application (but to organisms in the litter themselves). Consistent with this interpretation, the MAG abundance data did not indicate persistence of litter-associated taxa in soils or water samples at detectable levels. It should also be noted that the decay patterns of litter-associated taxa and the response of soil microorganisms to litter application are, in general, challenging to infer from time points (more frequent sampling will be required for this), and may also depend on environmental factors (e.g. intrinsic soil properties or weather and seasonal conditions, among others). Our sampling took place during the months of May–June, which are characterized by warm temperatures (20–30 °C) and frequent rain in the state of Georgia. Thus, weather conditions were highly favorable for (fast) microbial growth upon addition of exogenous carbon (litter) at our sampling sites, in general.

With respect to the antimicrobial resistance detection, independently of the approach used, no changes in the resistome of the amended soils and nearby watercourses could be identified by our study that could be associated to the impact of litter application. Although the MBL class of beta-lactamase genes was identified in notable abundance in the amended soils based on the ROcker model estimation, no pronounced differences were observed between these soils and the control soils. Therefore, the presence of MBL might be related to the indigenous resistome in soils rather than to the litter application itself (D'Costa et al., 2006; Wang et al., 2018). Congruent with this interpretation, other studies identified low ARG abundances or an insignificant increase in ARG concentrations in amended soils after short- and long-term litter application (Liu et al., 2021; Lopatto et al., 2019; Wang et al., 2018). However, there are several other studies whose findings contrast with those reported here and suggest that manure application on soils can introduce external ARGs and significantly increase their relative abundance in the soil environment (Han et al., 2018; Macedo et al., 2021; Xie et al., 2018; Zhang et al., 2017). Besides the increase, it has been suggested that the ARGs derived from manure do not persist for prolonged periods in the environment, likely due to the shift in the niche environment and competition by the indigenous microorganisms (Macedo et al., 2021; Tien et al., 2017; Zhang et al., 2017). Therefore, the longer time frames considered in the present study could account for the differences observed, and justify why only a few ARGs could be detected in our soil and water samples (see Table S4). Along the same line, the indigenous soil microbial diversity has also been suggested as an important source of resistance for invading bacterial pathogens carrying ARGs (Han et al., 2018), although further studies on the frequency that this occurs are still needed.

Overall, the discrepancies between our data and previous studies may be attributed to differences in the methodology, varying experimental designs with different time frames, intrinsic soil properties (e.g., physicochemical characteristics or complexity), and divergent seasonal or weather conditions. In this sense, future studies could include deeper sequencing of soil and water samples (allowing for higher community coverage and lower detection limits); shorter sampling time-frames after litter application in order to derive more robust decay curves; sampling of deeper soil layers in order to assess potential shifts in the deeper, anaerobic microbial communities; or the combined use of metagenomics with more sensitive techniques (e.g. ddPCR and isolation, allowing for lower detection limits and more precise counts). It is worth mentioning

that at the time the study was designed there was a lack of similar studies in the literature, and hence, limited information on the appropriate sampling time-frame to use. It is also important to note that our study did not assess chemical pollutants, possible effects at different depths, and the likelihood that chicken-derived taxa may persist at low abundances (rare biosphere) and/or as resting cells is possible. Therefore, our study cannot offer definitive conclusions about the overall safety of litter application. Despite these limitations, however, our findings are robust enough to suggest that at the studied CAFOs and weather conditions, litter application might represent a minimal impact on the microbiome and resistome of the natural soil and water microbial communities. The negligible effect on the microbiome or resistome profiles is probably due to the litter dilution in the field, and the recovery of the indigenous microbial community composition after a short-period of time, confirming the high resilience of natural microbial communities to perturbations.

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Author contributions

JB and KTK conceived the study and obtained funding; AD-V and BGL performed data analysis; AD-V produced the tables and figures; AL, RW and OG provided data and samples; JKH oversaw sequencing and provided data; AD-V and KTK wrote the first draft of the manuscript; all authors reviewed, edited and approved the manuscript.

CRedit authorship contribution statement

Ana Durán-Viseras: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Blake G. Lindner:** Writing – review & editing, Investigation. **Janet K. Hatt:** Writing – review & editing, Resources. **Amanda Lai:** Resources. **Robert Wallace:** Resources. **Olivia Ginn:** Resources. **Joe Brown:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Konstantinos T. Konstantinidis:** Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw sequence reads from each metagenome are publicly available in the SRA database under Bioproject PRJNA1047130.

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References

- Ayala-Ramirez, M., MacNell, N., McNamee, L.E., McGrath, J.A., Akhtari, F.S., Curry, M. D., Dunnon, A.K., Fessler, M.B., Garantzios, S., Parks, C.G., Fargo, D.C., Schmitt, C. P., Motsinger-Reif, A.A., Hall, J.E., Miller, F.W., Schurman, S.H., 2023. Association of distance to swine concentrated animal feeding operations with immune-mediated diseases: an exploratory gene-environment study. *Environ. Int.* 171, 107687 <https://doi.org/10.1016/j.envint.2022.107687>.
- Bolan, N.S., Szogi, A.A., Chuaavathi, T., Seshadri, B., Rothrock, M.J., Panneerselvam, P., 2010. Uses and management of poultry litter. *Worlds Poult. Sci. J.* <https://doi.org/10.1017/S0043933910000656>.
- Buchfink, B., Xie, C., Huson, D.H., 2015. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. <https://doi.org/10.1038/nmeth.3176>.
- Cantalapiedra, C.P., Hernández-Plaza, A., Letunic, I., Bork, P., Huerta-Cepas, J., 2021. eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol. Biol. Evol.* 38, 5825–5829. <https://doi.org/10.1093/molbev/msab293>.
- Castro, J.C., Rodríguez-R, L.M., Harvey, W.T., Weigand, M.R., Hatt, J.K., Carter, M.Q., Konstantinidis, K.T., 2018. imGLAD: accurate detection and quantification of target organisms in metagenomes. *PeerJ* 6, e5882. <https://doi.org/10.7717/peerj.5882>.
- Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2020. GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. *Bioinformatics* 36, 1925–1927. <https://doi.org/10.1093/bioinformatics/btz848>.
- Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *J. Environ. Qual.* 38, 1086–1108. <https://doi.org/10.2134/jeq2008.0128>.
- Cressman, M.D., Yu, Z., Nelson, M.C., Moeller, S.J., Lilburn, M.S., Zerby, H.N., 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Appl. Environ. Microbiol.* 76, 6572–6582. <https://doi.org/10.1128/AEM.00180-10>.
- D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., 2006. Sampling the antibiotic resistome. *Science* 311 (5759), 374–377. <https://doi.org/10.1126/science.1120800>.
- De Cesare, A., Caselli, E., Lucchi, A., Sala, C., Parisi, A., Manfreda, G., Mazzacane, S., 2019. Impact of a probiotic-based cleaning product on the microbiological profile of broiler litters and chicken caeca microbiota. *Poult. Sci.* 98, 3602–3610. <https://doi.org/10.3382/ps/pez148>.
- Dixon, P., 2003. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. <https://doi.org/10.1111/j.1654-1103.2003.tb02228.x>.
- Feng, Y., Wang, Y., Zhu, B., Gao, G.F., Guo, Y., Hu, Y., 2021. Metagenome-assembled genomes and gene catalog from the chicken gut microbiome aid in deciphering antibiotic resistomes. *Commun. Biol.* 4, 1305. <https://doi.org/10.1038/s42003-021-02827-2>.
- Florensa, A.F., Kaas, R.S., Clausen, P.T.L.C., Aytan-Aktug, D., Aarestrup, F.M., 2022. ResFinder – an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. *Microb. Genom.* 8, 000748 <https://doi.org/10.1099/mgen.0.000748>.
- Gilroy, R., Ravi, A., Getino, M., Pursley, I., Horton, D.L., Alkhan, N.F., Baker, D., Gharbi, K., Hall, N., Watson, M., Adriaenssens, E.M., Foster-Nyarko, E., Jarju, S., Secka, A., Antonio, M., Oren, A., Chaudhuri, R.R., La Razione, R., Hildebrand, F., Pallen, M.J., 2021. Extensive microbial diversity within the chicken gut microbiome revealed by metagenomics and culture. *PeerJ* 9, e10941. <https://doi.org/10.7717/peerj.10941>.
- Glendinning, L., Stewart, R.D., Pallen, M.J., Watson, K.A., Watson, M., 2020. Assembly of hundreds of novel bacterial genomes from the chicken caecum. *Genome Biol.* 21, 34. <https://doi.org/10.1186/s13059-020-1947-1>.
- Gonzales-Siles, L., Sjöling, Å., 2016. The different ecological niches of enterotoxigenic *Escherichia coli*. *Environ. Microbiol.* <https://doi.org/10.1111/1462-2920.13106>.
- Grenni, P., Patrolocco, L., Ademollo, N., Di Lenola, M., Barra Caracciolo, A., 2014. Capability of the natural microbial community in a river water ecosystem to degrade the drug naproxen. *Environ. Sci. Pollut. Res.* 21, 13470–13479. <https://doi.org/10.1007/s11356-014-3276-y>.
- Han, X.-M., Hu, H.-W., Chen, Q.-L., Yang, L.-Y., Li, H.-L., Zhu, Y.-G., Li, X.-Z., Ma, Y.-B., 2018. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102. <https://doi.org/10.1016/j.soilbio.2018.08.018>.
- Henchion, M., Moloney, A.P., Hyland, J., Zimmermann, J., McCarthy, S., 2021. Review: trends for meat, milk and egg consumption for the next decades and the role played by livestock systems in the global production of proteins. *Animal* 15, 100287. <https://doi.org/10.1016/j.animal.2021.100287>.
- Hu, H.-W., Han, X.-M., Shi, X.-Z., Wang, J.-T., Han, L.-L., Chen, D., He, J.-Z., 2016. Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure. *FEMS Microbiol. Ecol.* 92, fiv169. <https://doi.org/10.1093/femsec/fiv169>.
- Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S.K., Cook, H., Mende, D.R., Letunic, I., Rattei, T., Jensen, L.J., von Mering, C., Bork, P., 2019. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* 47, D309–D314. <https://doi.org/10.1093/nar/gky1085>.
- Hyatt, D., Chen, G.-L., Locascio, P.F., Land, M.L., Larimer, F.W., Hauser, L.J., 2010. Motif: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11, 119. <https://doi.org/10.1186/1471-2105-11-119>.
- Jechalke, S., Focks, A., Rosendahl, I., Groeneweg, J., Siemens, J., Heuer, H., Smalla, K., 2014. Structural and functional response of the soil bacterial community to

- application of manure from difloxacin-treated pigs. *FEMS Microbiol. Ecol.* 87, 78–88. <https://doi.org/10.1111/1574-6941.12191>.
- Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., Wang, Z., 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 2019, e7359. <https://doi.org/10.7717/peerj.7359>.
- Kreling, V., Falcone, F.H., Kehrenberg, C., Hensel, A., 2020. *Campylobacter* sp.: pathogenicity factors and prevention methods-new molecular targets for innovative antivirulence drugs? *Appl. Microbiol. Biotechnol.* 104, 10409–10436. <https://doi.org/10.1007/s00253-020-10974-5> [Published].
- Lindner, B.G., Suttner, B., Zhu, K.J., Conrad, R.E., Rodriguez-R, L.M., Hatt, J.K., Brown, J., Konstantinidis, K.T., 2022. Toward shotgun metagenomic approaches for microbial source tracking sewage spills based on laboratory mesocosms. *Water Res.* 210, 117993 <https://doi.org/10.1016/j.watres.2021.117993>.
- Liu, C., Chen, Y., Li, X., Zhang, Y., Ye, J., Huang, H., Zhu, C., 2020. Temporal effects of repeated application of biogas slurry on soil antibiotic resistance genes and their potential bacterial hosts. *Environ. Pollut.* 258, 113652 <https://doi.org/10.1016/j.envpol.2019.113652>.
- Liu, W., Ling, N., Guo, J., Ruan, Y., Wang, M., Shen, Q., Guo, S., 2021. Dynamics of the antibiotic resistome in agricultural soils amended with different sources of animal manures over three consecutive years. *J. Hazard. Mater.* 401, 123399 <https://doi.org/10.1016/j.jhazmat.2020.123399>.
- Lo, C.-C., Chain, P.S., 2014. Rapid evaluation and quality control of next generation sequencing data with FaQCs. *BMC Bioinformatics* 15, 366.
- Lopatto, E., Choi, J., Colina, A., Ma, L., Howe, A., Hinsale-Leasure, S., 2019. Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. *PLoS One* 14, e0220770. <https://doi.org/10.1371/journal.pone.0220770>.
- Lopes, A.R., Faria, C., Prieto-Fernández, A., Trasar-Cepeda, C., Manaia, C.M., Nunes, O. C., 2011. Comparative study of the microbial diversity of bulk paddy soil of two rice fields subjected to organic and conventional farming. *Soil Biol. Biochem.* 43, 115–125. <https://doi.org/10.1016/j.soilbio.2010.09.021>.
- Lu, J., Breitwieser, F.P., Thielen, P., Salzberg, S.L., 2017. Bracken: estimating species abundance in metagenomics data. *PeerJ Comput. Sci.* 3, e104 <https://doi.org/10.7717/peerj-cs.104>.
- Lynch, M.D.J., Neufeld, J.D., 2015. Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* <https://doi.org/10.1038/nrmicro3400>.
- Macedo, G., van Veen, H.P.J., Hernandez-Leal, L., van der Maas, P., Heederik, D., Mevius, D., Bossers, A., Schmitt, H., 2021. Targeted metagenomics reveals inferior resilience of farm soil resistome compared to soil microbiome after manure application. *Sci. Total Environ.* 770, 145399 <https://doi.org/10.1016/j.scitotenv.2021.145399>.
- Mallin, M.A., McIver, M.R., 2018. Season matters when sampling streams for swine CAFO waste pollution impacts. *J. Water Health* 16, 78–86. <https://doi.org/10.2166/wh.2017.194>.
- Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ. Pollut.* 157, 2893–2902. <https://doi.org/10.1016/j.envpol.2009.05.051>.
- Mikheenko, A., Saveliev, V., Gurevich, A., 2016. MetaQUAST: evaluation of metagenome assemblies. *Bioinformatics* 32, 1088–1090. <https://doi.org/10.1093/bioinformatics/btv697>.
- Mull, B., Hill, V.R., 2012. Recovery of diverse microbes in high turbidity surface water samples using dead-end ultrafiltration. *J. Microbiol. Methods* 91, 429–433. <https://doi.org/10.1016/j.mimet.2012.10.001>.
- National Agricultural Statistics Service, 2022. Poultry-Production and Value.
- National Chicken Council, 2021. Broiler chicken industry key facts 2021 [WWW document]. <https://www.nationalchickencouncil.org/about-the-industry/statistics/broiler-chicken-industry-key-facts/>.
- Nayfach, S., Pollard, K.S., 2015. Average genome size estimation improves comparative metagenomics and sheds light on the functional ecology of the human microbiome. *Genome Biol.* 16, 51. <https://doi.org/10.1186/s13059-015-0611-7>.
- Olm, M.R., Brown, C.T., Brooks, B., Banfield, J.F., 2017. DRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. *ISME J.* 11, 2864–2868. <https://doi.org/10.1038/ismej.2017.126>.
- Ondov, B.D., Treangen, T.J., Melsted, P., Mallonee, A.B., Bergman, N.H., Koren, S., Phillippy, A.M., 2016. Mash: fast genome and metagenome distance estimation using MinHash. *Genome Biol.* 17, 132. <https://doi.org/10.1186/s13059-016-0997-x>.
- Orellana, L.H., Rodriguez-R, L.M., Konstantinidis, K.T., 2016. ROCKER: accurate detection and quantification of target genes in short-read metagenomic data sets by modeling sliding-window bitscores. *Nucleic Acids Res.* 45, e14 <https://doi.org/10.1093/nar/gkw900>.
- Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* 25, 1043–1055. <https://doi.org/10.1101/gr.186072.114>.
- Peng, Y., Leung, H.C.M., Yiu, S.M., Chin, F.Y.L., 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* 28, 1420–1428. <https://doi.org/10.1093/bioinformatics/bts174>.
- Poulsen, P.H.B., Al-Soud, W.A., Bergmark, L., Magid, J., Hansen, L.H., Sørensen, S.J., 2013. Effects of fertilization with urban and agricultural organic wastes in a field trial – prokaryotic diversity investigated by pyrosequencing. *Soil Biol. Biochem.* 57, 784–793. <https://doi.org/10.1016/j.soilbio.2011.12.023>.
- Prijbelski, A., Antipov, D., Meleshko, D., Lapidus, A., Korobeynikov, A., 2020. Using SPAdes de novo assembler. *Curr. Protoc. Bioinformatics* 70, e102. <https://doi.org/10.1002/cpbi.102>.
- Qu, A., Brulc, J.M., Wilson, M.K., Law, B.F., Theoret, J.R., Joens, L.A., Konkel, M.E., Angly, F., Dinsdale, E.A., Edwards, R.A., Nelson, K.E., White, B.A., 2008. Comparative metagenomics reveals host specific metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS One* 3, e2945. <https://doi.org/10.1371/journal.pone.0002945>.
- Riber, L., Poulsen, P.H.B., Al-Soud, W.A., Skov Hansen, L.B., Bergmark, L., Brejnrod, A., Norman, A., Hansen, L.H., Magid, J., Sørensen, S.J., 2014. Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. *FEMS Microbiol. Ecol.* 90, 206–224. <https://doi.org/10.1111/1574-6941.12403>.
- Rieke, E.L., Soupir, M.L., Moorman, T.B., Yang, F., Howe, A.C., 2018. Temporal dynamics of bacterial communities in soil and leachate water after swine manure application. *Front. Microbiol.* 9, 3197. <https://doi.org/10.3389/fmicb.2018.03197>.
- Ritchie, H., Rosado, P., Roser, M., 2017. Meat and Dairy Production. *Our World in Data*.
- Rodriguez-R, L.M., Konstantinidis, K.T., 2014. Estimating coverage in metagenomic data sets and why it matters. *ISME J.* <https://doi.org/10.1038/ismej.2014.76>.
- Rodriguez-R, L.M., Konstantinidis, K.T., 2016. The envomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Prepr.* <https://doi.org/10.7287/peerj.preprints.1900v1>.
- Rodriguez-R, L.M., Gunturu, S., Harvey, W.T., Rosselló-Mora, R., Tiedje, J.M., Cole, J.R., Konstantinidis, K.T., 2018. The Microbial Genomes Atlas (MiGA) webserver: taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome level. *Nucleic Acids Res.* 46, W282–W288. <https://doi.org/10.1093/nar/gky467>.
- Segura-Wang, M., Grabner, N., Koestelbauer, A., Klose, V., Ghanbari, M., 2021. Genome-resolved metagenomics of the chicken gut microbiome. *Front. Microbiol.* 12, 726923 <https://doi.org/10.3389/fmicb.2021.726923>.
- Steinegger, M., Söding, J., 2017. MMseqs2 enables sensitive protein sequence searching for the analysis of massive data sets. *Nat. Biotechnol.* 35, 1026–1028. <https://doi.org/10.1038/nbt.3988>.
- Suttner, B., Johnston, E.R., Orellana, L.H., Rodriguez-R, L.M., Hatt, J.K., Carychao, D., Carter, M.Q., Cooley, M.B., Konstantinidis, K.T., 2020. Metagenomics as a public health risk assessment tool in a study of natural creek sediments influenced by agricultural and livestock runoff: potential and limitations. *Appl. Environ. Microbiol.* 86, e02525–e2619. <https://doi.org/10.1128/AEM.02525-19>.
- Tien, Y.-C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017. Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. *Sci. Total Environ.* 581–582, 32–39. <https://doi.org/10.1016/j.scitotenv.2016.12.138>.
- U.S. Environmental Protection Agency, 2001. Development Document for the Proposed Revisions to the National Pollutant Discharge Elimination System Regulation and the Effluent Guidelines for Concentrated Animal Feeding Operations.
- Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci. U. S. A.* 112, 5649–5654. <https://doi.org/10.1073/pnas.1503141112>.
- Wang, F., Xu, M., Stedtfeld, R.D., Sheng, H., Fan, J., Liu, M., Chai, B., Soares De Carvalho, T., Li, H., Li, Z., Hashsham, S.A., Tiedje, J.M., 2018. Long-term effect of different fertilization and cropping systems on the soil antibiotic resistome. *Environ. Sci. Technol.* 52, 13037–13046. <https://doi.org/10.1021/acs.est.8b04330>.
- Wang, L., Oda, Y., Grewal, S., Morrison, M., Michel, F.C., Yu, Z., 2012. Persistence of resistance to erythromycin and tetracycline in swine manure during simulated composting and lagoon treatments. *Microb. Ecol.* 63, 32–40. <https://doi.org/10.1007/s00248-011-9921-9>.
- Wang, L., Lilburn, M., Yu, Z., 2016. Intestinal microbiota of broiler chickens as affected by litter management regimens. *Front. Microbiol.* 7, 593. <https://doi.org/10.3389/fmicb.2016.00593>.
- Wang, M., Sun, Y., Liu, P., Sun, J., Zhou, Q., Xiong, W., Zeng, Z., 2017. Fate of antimicrobial resistance genes in response to application of poultry and swine manure in simulated manure-soil microcosms and manure-pond microcosms. *Environ. Sci. Pollut. Res.* 24, 20949–20958. <https://doi.org/10.1007/s11356-017-9623-z>.
- Wickham, H., 2011. ggplot2. *Wiley Interdiscip. Rev. Comput. Stat.* 3, 180–185. <https://doi.org/10.1002/wics.147>.
- Wood, D.E., Lu, J., Langmead, B., 2019. Improved metagenomic analysis with Kraken 2. *Genome Biol.* 20, 257. <https://doi.org/10.1186/s13059-019-1891-0>.
- Wu, Y.W., Simmons, B.A., Singer, S.W., 2016. MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* 32, 605–607. <https://doi.org/10.1093/bioinformatics/btv638>.
- Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K., Yang, H., 2017. Microbial community mapping in intestinal tract of broiler chicken. *Poult. Sci.* 96, 1387–1393. <https://doi.org/10.3382/ps/pew372>.
- Xie, W.Y., Yuan, S.T., Xu, M.G., Yang, X.P., Shen, Q.R., Zhang, W.W., Su, J.Q., Zhao, F.J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. *Soil Biol. Biochem.* 122, 111–119. <https://doi.org/10.1016/j.soilbio.2018.04.009>.
- Yang, J., Tong, C., Xiao, D., Xie, L., Zhao, R., Huo, Z., Tang, Z., Hao, J., Zeng, Z., Xiong, W., 2022. Metagenomic insights into chicken gut antibiotic resistomes and microbiomes. *Microbiol. Spectr.* 10, e0190721 <https://doi.org/10.1128/spectrum.01907-21>.
- Zhang, S.-Y., Suttner, B., Rodriguez-R, L.M., Orellana, L.H., Conrad, R.E., Liu, F., Rowell, J.L., Webb, H.E., Williams-Newkirk, A.J., Huang, A., Konstantinidis, K.T., 2022. ROCKER models for reliable detection and typing of short-read sequences

- carrying β -lactamase genes. *mSystems* 7, e0128121. <https://doi.org/10.1128/msystems.01281-21>.
- Zhang, X.-X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* 82, 397–414. <https://doi.org/10.1007/s00253-008-1829-z>.
- Zhang, Y.J., Hu, H.W., Gou, M., Wang, J.T., Chen, D., He, J.Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. *Environ. Pollut.* 231, 1621–1632. <https://doi.org/10.1016/j.envpol.2017.09.074>.