agriculture

MDPI

Article

The Impact Aerobic and Anaerobic Incubations of Poultry Litter Have on Class 1 Integron Resistome and Microbiome

John J. Maurer [1,*], Alexa Hoke [1], Keshav C. Das [2], Jian Wu [2], Mark A. Williams [3], Sydney Kinstler [1], Casey Ritz [4], Gregory P. Pittman [5], Roy Berghaus [6] and Margie D. Lee [7]

1 School of Animal Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA; alexa.hoke@gmail.com (A.H.); stuckwiller@neogen.com (S.K.)
2 College of Engineering, University of Georgia, Athens, GA 30602, USA; kdas@uga.edu (K.C.D.); jian.wu1@uga.edu (J.W.)
3 School of Plant and Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA; markwill@vt.edu
4 Department of Poultry Science, University of Georgia, Athens, GA 30602, USA; critz@uga.edu
5 Cooperative Extension Service, University of Georgia, Athens, GA 30602, USA; gpittman@uga.edu
6 Department of Population Health, University of Georgia, Athens, GA 30602, USA; berghaus@uga.edu
7 Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, USA; mlee2@vt.edu
* Correspondence: jjmaurer@vt.edu

Abstract: Animal manure is a desirable fertilizer because of its rich nitrogen, but it also contains a large and diverse reservoir of antimicrobial resistance (AMR) genes (ARGs). To reduce this AMR reservoir, five treatments (passive aeration, forced aeration, static or anaerobic incubations, autoclaving) were assessed for their impact on the poultry litter resistome. Bacterial DNA was extracted from the litter and the qPCR-estimated copy number of 16S *rrs*, class1 integrons (*intI1*) and associated resistance genes (*aadA*, *sul1*). Then, 16S amplicon metagenomic sequencing was used to determine community diversity and composition. Depending on incubation conditions, class 1 integrons and their associated ARGs were reduced by 0.5 to 1.0 Log_{10}/g poultry litter. Only autoclaving reduced integrons and associated AMR genes by three Log_{10}. Changes in AMR abundance reflected fluctuations in litter bacteriome composition at the family, genus, and sequence variant level. There was a negative correlation between class 1 integron and AMR genes, with genera belonging to Actinobacteria, Firmicutes, and Proteobacteria phyla. While these poultry litter treatments failed to reduce AMR abundance, aerobic and anaerobic treatments reduced taxons that contained pathogenic species. The approach to remediating resistance in poultry litter may be more effective if is focused on reducing bacterial pathogens.

Keywords: poultry litter; resistome; class 1 integron

Received: 5 December 2024
Revised: 9 February 2025
Accepted: 11 February 2025
Published: 13 February 2025

Citation: Maurer, J.J.; Hoke, A.; Das, K.C.; Wu, J.; Williams, M.A.; Kinstler, S.; Ritz, C.; Pittman, G.P.; Berghaus, R.; Lee, M.D. The Impact Aerobic and Anaerobic Incubations of Poultry Litter Have on Class 1 Integron Resistome and Microbiome. *Agriculture* **2025**, *15*, 398. https://doi.org/10.3390/agriculture15040398

1. Introduction

There are considerable environmental concerns associated with the use of animal manure as a soil amendment. Application of animal manures to fields has some public health risks [1], as these manures may contain zoonotic pathogens including Salmonella, enterohemorrhagic *Escherichia coli*, *Campylobacter*, *Listeria monocytogenes* and *Cryptosporidium parvum* [2–4]. Over the past 40 years, there has been a significant increase in foodborne outbreaks associated with the consumption of fruits, nuts and vegetables [5–7]. Several of these outbreaks have been tied directly to the application of animal manures to fields [8] or caused indirectly, through the contamination of irrigation water [9,10]. In addition, there is

concern that run-off from animal manure-amended soils will contaminate drinking water. Poultry litter has a high phosphate content that, if washed into ponds, lakes or streams, will contribute to the eutrophication and death of aquatic life in these water bodies [11,12].

A complex microbial community inhabits poultry litter, consisting of more than fecal bacteria. Gram-positives are the dominant population, containing member species belonging to the families *Lactobacillaceae*, *Aerococcaceae*, *Carnobacteriaceae*, *Staphylococcaceae*, *Corynebacteriaceae*, and *Micrococcaceae* [13]. In addition to pathogens, animal manures also contain a diverse and highly abundant antimicrobial resistome [14]. The sizable resistome [1,15] includes integrons, genetic elements that capture and build antimicrobial resistance assemblages [16] and their associated resistance genes. There are four classes of integrons consisting of a site-specific recombinase or integrase (*intI*) and an integration site (*attI*). The different integron classes (*intI1-4*) are defined by the homology of the *intI* sequences and the conserved gene sequences, 3', to the integration site. These integrons contain antimicrobial resistance genes (ARGs) inserted into the *attI* integration site [17]. Class 1 integrons consist of an integrase, IntI1, an *attI1* integration site into which antimicrobial resistance genes (ARGs) are integrated and the conserved ARG *sul1*, is inserted 3'to the integration site. The ARG inserted into the *attI1* varies, is diverse [18], and may contain multiple ARGs [19]. In multi-drug-resistant (MDR) *Escherichia coli* isolated from poultry, most isolates contain class 1 integrons, and the streptomycin resistance gene *aadA1* is commonly found within its *attI* integration site [20]. *intI1* and *sul1* are abundant in the poultry litter microbiome, where as much as 11% of the total bacterial population possess these genes. Class 2 integrons are a minor component of the poultry litter resistome, present at levels comparable to the Gram-negative *Enterobacteriaceae* population in litter. These class 1 integrons are present in the dominant Gram-positive population of poultry litter, and many of these Gram-positives isolates also possess the streptomycin-resistance gene *aadA* in the *aatI1* integration site [1]. There is significant concern over spillover of these resistances into soil and plant microbiomes when applying manures to crop soils. There are numerous studies that have documented the adverse effects of manure applications on the soil [21–23] and plant resistomes [24–26]. Most concerning is the acquisition of these resistances by pathogens and their potential spill over into the human population, which would adversely affect infection control and result in treatment failure. The best way to prevent this is to identify a treatment process that reduces the abundance of ARGs in animal manures.

The United States produces 9.18 billion broiler chickens, 394 million layer-chickens, and 238 million turkeys each year, which translates into~twenty million tons of poultry manure annually [27,28]. Birds are raised on wood shavings and similar absorptive products, on which the animals defecate. Poultry litter under correct management is partially responsible for good animal production indices [29], because it acts as a source of beneficial organisms to colonize the intestinal tract of neonatal poultry in the absence of direct exposure to the microbiota of adult birds [30].

Animal manures are often applied to soil in cropping systems to enrich soil organic matter and provide plants with essential nutrients [31]. Manures are typically applied to meet phosphorous and nitrogen needs for plant growth. Other essential nutrients are also found in manures, which when applied to soil often result in elevated levels of macro- and micronutrients such as sulfur and zinc [32]. Manures are compatible with organic produce production, which, for many countries, has been the most affordable way to improve soil and minimize erosion (available online: https://www.ams.usda.gov/grades-standards/soil-building-manures-composts). Poultry litter has high levels of nutrients, especially ammonium, that make it an effective and affordable fertilizer [11,28]. How can animal

manure like poultry litter be remediated of pathogens or antimicrobial resistance while maintaining its nutrient concentration as a soil amendment for growing crops?

Composting is the biological stabilization of organic waste. When placed in large piles, the solid matrix becomes self-insulated and promotes microbial activity that creates thermophilic conditions (55–65 °C). The retained heat, over time, kills pathogens, while the active thermophiles consume easily digestible organics, making the compost biologically more stable. Typically, the composting matrix is mixed periodically to homogenize its contents. Compost piles are either created with a geometry that enhances natural aeration or kept aerobic through forced aeration. Over a period of days, the easily available organics are degraded, and the compost becomes stable with declining temperature and bacterial populations. Litter management is important in controlling poultry disease within the production industry [33]. Between flock placements, the resulting poultry litter is piled into rows along the length of the poultry house and allowed to set before it is spread out across the surface of the house and top-dressed with fresh bedding. This process, referred to as windrow processing, generates heat from the microbial activity inherent in composting, to kill pathogens [34].

Numerous studies have evaluated composting to reduce antibiotic concentrations, antibiotic-resistant bacteria and antibiotic-resistance gene abundance in swine and dairy manures [35–39]. Aerobic composting has been shown to be effective at reducing tetracyclines and macrolide residues, but ineffective for sulfonamides in animal manures [35,38,40]. The success of composting at reducing ARG has been variable depending on the antibiotic resistance gene studied [39,41]. Internal temperatures generated during composting are critical [42] and carbon amendment also facilitates the decrease in antibiotic resistance [43] and pathogen abundance [44,45]. There has also been some success with anaerobic digestion at reducing antibiotic residues and resistance gene abundance in animal and human waste [46–48]. Anaerobic digestion has the added benefit of retaining nitrogen content in manures as well as producing the by-product methane, a marketable energy source.

There is strong evidence that animal and human feces significantly impact the environmental resistome [49–52]. Can composting animal manures like poultry litter reduce ARG load? Do changes in the bacteriome affect ARG load? Most importantly, what composting conditions can reduce ARG load without compromising the nutrient availability of poultry litter as a soil amendment for plant growth? Few studies have examined the impact of composting poultry litter on its ARG load. In those studies, a single incubation condition, aeration, was examined [53,54] and one included poultry litter amendments [54]. Neither study examined the impact of composting on the nutrient concentration of composted chicken litter. This study assessed five poultry litter composting treatments, including steam explosion (autoclaving), for reducing antimicrobial resistances associated with integrons. Differences in the antimicrobial resistance gene load, in response to litter conditions, reflected changes in the poultry litter bacteriome. None of the incubation conditions examined in this study adversely affected the nutrient concentration of the composted poultry litter as a soil amendment for plant growth.

2. Materials and Methods

2.1. Bench Scale Aerobic and Anaerobic Treatment of Poultry Litter

Different poultry litter incubation conditions were examined that would modulate the bacterial composition. Would the resulting changes in community structure decrease class 1 integron and associated ARG levels in poultry litter? A culture-independent approach was used to assess changes in ARG levels and bacterial community composition.

Ten gallons of poultry litter was collected 18 July 2018, from a stack house [55] in Madison, Georgia, storing litter from a commercial poultry house cleaned out two weeks

prior. Pine shavings are typically used as the bedding material, found in poultry litter, for poultry production in the southeastern United States. Poultry litter was stored at −18 °C before use. For incubation experiments, litter (1.8 kg) was rehydrated with 1.8 L of deionized H_2O to produce reactor material with 55% water content. Poultry litter was homogenized, and 100 g wet weight was prepared for incubation. Treatments were as follows. Passive aeration (T1) involved placement of samples into 200 mL beakers. To simulate forced-aerated composting (T2), poultry litter samples were placed in 250 mL Erlenmeyer flasks receiving a tube delivering a continuous flow of air above the litter. Anaerobic digestion (T3) involved placing samples into 250 mL serum bottles, sealed with a rubber stopper, and gassed with nitrogen. Finally, the static treatment (T4) consisted of litter in covered beakers, which served to simulate stack house storage of poultry litter. Treatments T1, T2 and T3 were maintained in an incubator (Innova 400 Incubator-Shaker, New Brunswick Scientific; Edison, NJ) at 45 °C for 28 days (Figure 1), while T4 was maintained at room temperature (25 °C). This incubation temperature was chosen based on prior knowledge that mesophilic conditions around 45 °C provide for maximal biological degradation of plant material [56]. Each of the four treatments was replicated eight times, translating to 32 experimental units.

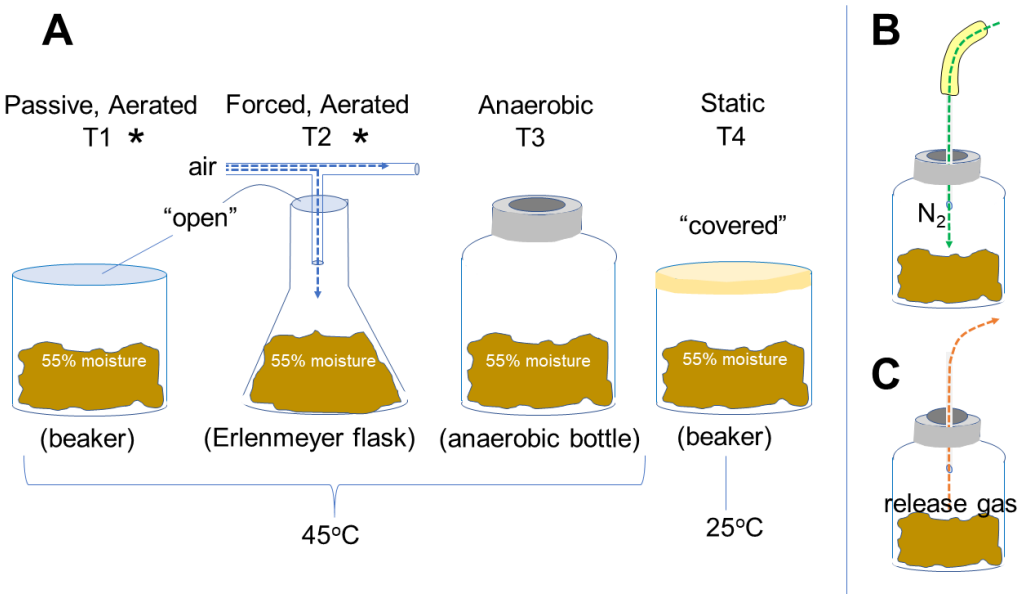

Figure 1. Bench scale aerobic and anerobic poultry litter treatments. Poultry litter was rehydrated to 55% water content. Different incubation conditions (**A**) were examined, including passive aeration (T1), forced aeration (T2), anaerobic (T3), and a static treatment condition (T4). To create anaerobic conditions, the poultry sample was flushed with nitrogen at the beginning of incubation (**B**). Collected gases in this anerobic system were released daily (**C**). Incubation treatments T1, T2, and T3 were performed at 45 °C for 28 days, while T4 was maintained at room temperature (25 °C). Water was replenished daily in reactors T1 and T2 to maintain 55% water content in litter material (*).

Forced aeration treatments (T2) were continuously flushed with room air at a flow rate more than 0.73 $L_{air}/L_{\text{reactor volume}}/d$. This level of aeration is sufficiently higher than the design oxygen requirements for aerobic composting (e.g., Chapter 9 describes a Newberg OR in-vessel composting plant where the design aeration specification is 0.086 L/L-d) [57]. This flow rate would also ensure that oxygen levels always remained above anaerobic thresholds of 6% [58]. Anaerobic conditions (T3) were produced by sparging bottles with nitrogen gas (100%) at the beginning of incubation. Daily gas build-up, associated with microbial activity, was released from anaerobic bottles daily by puncturing the rubber stopper with a 20-gauge needle.

The reactors (T1 and T2) were removed from the incubator once a day and weighed. Loss of weight was primarily attributed to drying alone, and therefore water was added back to the reactor to replenish the moisture to 55% and returned to the incubator. Daily addition of water was not performed in treatments T3 and T4 as there were no large weight changes relative to T1 and T2. Incubation was terminated after 28 days when the samples were removed from the flask and split into two. The duration was selected based on past experiences in composting poultry litter. Typically, the first heat cycle is completed in 15–20 days. With poultry litter being an easily decomposable substrate, at optimal conditions of temperature and moisture, completion of the initial, most-active phase of composting can be anticipated. An additional week provides enough time to complete these processes [59]. One sample was immediately frozen (−18 °C) and kept frozen until it was transferred to Virginia Tech. The second sample was used to estimate final water content, and subsequently ground and analyzed for total C, N, and ash contents. The moisture and ash content of samples were measured using standard methods outlined in the Test Methods for Evaluation of Composting and Compost Products [60]. Ground samples (less than 250 um) were analyzed for CHN using 3 mg of each sample in a Mirco-Dumas Combustion Analyzer, where complete combustion transfers elements to the gas phase and is subsequently measured using a C/H/N Analyzer, Model NA 1500 (Carlo Erba Strumentazione; Milan, Italy). The analyzer is calibrated by including solid-phase reference materials at the beginning of each run and at fixed intervals thereafter. Ultra-high-purity acetanilide is the most frequently used standard material.

Poultry litter (12 g) subsamples were placed in a 200 mL capacity beaker with aluminum foil covering the top and autoclaved at 121 °C, 15 psi, for 10 min on a gravity cycle (total autoclave time: 30 min) (STERIS Amsco Century Sg-120 Gravity Steam Sterilizer Autoclave; Steric Corporation; Mentor, OH, USA). Total aerobic plate counts were determined for starting and autoclaved poultry litter material as follows. Bacterial were extracted from 1 g of litter using a gyratory shaker and spin columns, as detailed by Oxendine et al. [61]. Suspensions were diluted 10-fold in buffered saline gelatine [62], plated on Tryptic Soy Agar (Difco; Detroit, MI, USA) (10^{-2} to 10^{-7} final dilution) and incubated aerobically overnight at 37 °C. Total DNA was extracted from autoclaved poultry litter as detailed below.

2.2. DNA Extraction and qPCR

The starting poultry litter served as an untreated control in comparison with the different treatments. DNA was extracted from samples (0.25 g) using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research; Irvine, CA, USA). The kit was used according to the manufacturer's instructions, vortexing bacterial suspensions with beads for 10 min. DNA was quantified using a NanoDrop One (Thermo Fisher Scientific; Waltham, MA, USA). Aliquots of DNA were made in fresh tubes and were normalized to 10 ng/µL using molecular-grade deionized H_2O. DNA samples were stored at −20 °C. qPCR was used to estimate total gene copy number for 16S rRNA and streptomycin-resistance, sulfonamide-resistance, and class 1 integron-integrase genes *aadA*, *sul1*, and *intI1*, respectively, in the poultry litter community (Table 1). *sul1* was chosen based on its position and conservation in class 1 integrons and its high abundance, at levels superimposable with intI1 in poultry litter [1]. The aminoglycoside resistance gene *aadA1* was selected based on its presence within the class 1 integron' s *attI1* integration site of poultry *Escherichia coli* [20] and abundant Gram-positive bacteria in poultry litter [1]. *Escherichia coli* pDU2020 served as a positive control for all PCR primers. A standard curve was generated using *E. coli* pDU202 and a series of ten-fold dilutions. qPCR mixtures contained 5 µL of iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), 0.5 µM of forward and reverse primers, 1 µL of template DNA, and molecular grade water for a final volume of 10 µL. The following thermocycler (Bio-Rad CFX96 Real Time

System; C1000 Touch TM thermocycler) conditions were used: 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at an annealing temperature, and 2 min at 72 °C. This was followed by a melt curve with a temperature range of 55 to 95 °C at 0.5 °C increments. The melting curves were used to confirm the identity of the sample signal as the target gene in question if its melting peak profile overlapped with the positive PCR control. The exception to this was 16S, where all litter and treatment samples produced the same, distinct melting curve compared to *E. coli* DH5α and pDU202, PCR controls. Poultry litter is commonly dominated by Firmicutes [13].

Table 1. PCR primers.

Target Gene [1]	Sequence	Amplicon Size (bp)	Annealing Temp (°C)	Reference
16S *rrs*	F: CGGTGAATACGTTCYCGG R: GGWTACCTTGTTACGACTT	142	56.3	[1]
aadA1	F: GTACGGCTCCGCAGTGGA R: GCGCTGCCATTCTCCAAA	244	56.3	[1]
sul1	F: TTGGGGCTTCCGCTATTGGTCT R: GGGTTTCCGAGAAGGTGATTGC	187	62.0	[1]
intI1	F: CCTCCCGCACGATGATC R: TCCACGCATCGTCAGGC	280	56.3	[1]

[1] *Escherichia coli* with pDU202 serving as a positive control for all PCR primers.

2.3. The 16 S Amplicon Metagenomic Sequencing

DNA samples extracted with ZymoBiomics DNA Miniprep Kit were sent to Zymo Research for 16S sequencing. The Quick-16S NGS Library Prep Kit (Zymo Research, Irvine, CA, USA) was used for 16S *rrs* targeted sequencing. PCR was used to prepare the sequencing library using V3 16S primers, and Select-a-Size DNA Clean & Concentrator (Zymo Research, Irvine, CA, USA) was used to clean the final pooled library. Sequencing of the final library took place on Illumina MiSeq.

2.4. Bioinformatics

Bioinformatics analysis was performed by Zymo Research. Samples were demultiplexed and preprocessed using the consensus method in DADA2 [63] and filtered through cutadapt version 1.18 [64] using the following parameters: minimum length = 150 bp, maximum length = 250 bp, phred quality score > 30. Reads were merged using fastq-join. A further filtering step through cutadapt dropped merged amplicons lengths < 200 bp and >290 bp. This was followed by removing chimeric reads using USEARCH version 6.1 [65]. Further processing was performed in QIIME 2 [66] using the pick_open_reference_otus.py script. Reads were clustered into sequence variants (SVs) at a 100% threshold using UCLUST [65]. SV taxonomy assignments were based on Greengenes database version 13.8 [67]. The SVs related to mitochondrial and plastidial 16S rRNA genes were removed. To reduce SV inflation, SVs matching the following criteria were removed: spurious SVs that had just one read in all samples, SVs that were present in less than three samples, and SVs with less than 10 total reads. The remaining SVs were retained for downstream analyses. All 16S rRNA nucleotide sequences were archived in NCBI under BioProject PRJNA1082344.

2.5. Statistical Analyses

Box plots of qPCR results were created in RStudio (Boston, MA, USA). *t*-tests were performed in Excel to determine statistical significance between treated and untreated litter samples. Paired student *t*-tests were run using the two-tailed distribution and considered significant for *p*-values < 0.05. Standard curves created with *E. coli* pDU202 were used to estimate the gene copy number of 16S rRNA, *aadA*, *sul1*, and *intI1* within samples. Copy

numbers per sample were then used to calculate the copy number per gram of litter. Copy numbers were Log_{10} transformed into Excel. Boxplots of antimicrobial resistance genes per gram of litter were created in RStudio (Boston, MA, USA). Pearson's correlation was used to assess association between bacterial taxa percentages with *intI1*, *sul1* or *aadA1* abundance. Values were considered significant for r values > 0.6999 or <−0.6999 with *p*-values < 0.05.

3. Results

3.1. Impact of Poultry Litter Incubation Processes on Class 1 Integron and Abundance of Associated Antimicrobial Resistance Genes aadA1 and sul1

Altering the incubation conditions had a significant effect on *aadA1* and *sul1* gene abundance. Anaerobiosis reduced the abundance of the streptomycin gene *aadA1* by 0.50 Log_{10} ($p < 0.01$), but forced aeration increased its abundance by 0.41 Log_{10} ($p < 0.01$). The sulfonamide resistance gene *sul1* was also significantly reduced by 0.50 Log_{10} ($p < 0.01$) with static and anaerobic incubations. However, none of the treatments significantly reduced *intI1*, a marker for class 1 integron, in any of the samples (*p*-values > 0.05), apart from autoclaving, which reduced its abundance and the other integron-associated antibiotic resistance genes *sul1* and *aadA1* 2.00–3.00 Log_{10} per gram of poultry litter ($p < 0.01$) (Figure 2).

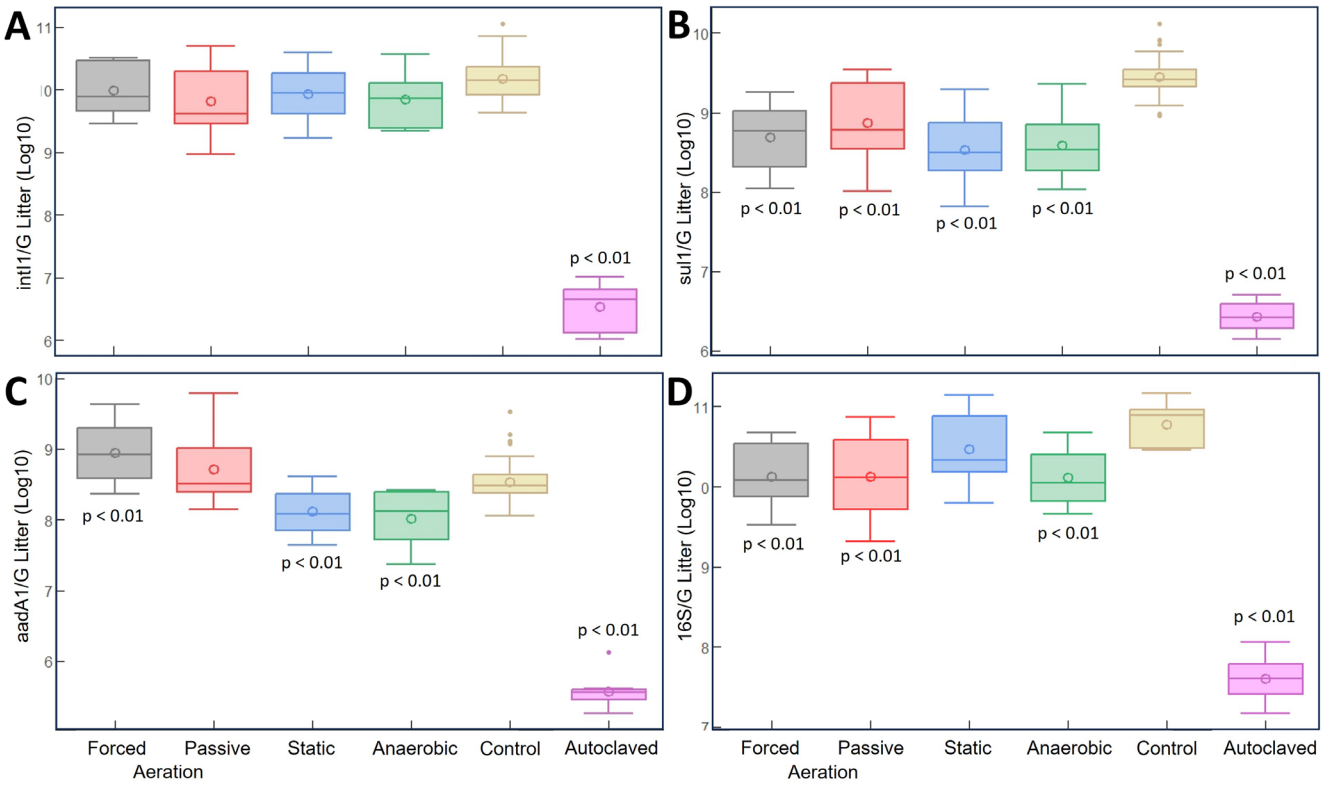

Figure 2. Class one integron (**A**), associated antimicrobial resistance *aadA1* (**B**) and *sul1* (**C**) gene abundance and (**D**) bacterial genomes (16S rRNA) in poultry litter before (control) and after various incubation conditions. qPCR was used to quantify bacterial genomes (16S rRNA) and genes (*intI1*, *aadA1* and *sul1*). DNA quantities were normalized to 10 ng per PCR reaction. *Escherichia coli* with pDU202 served as positive controls and known DNA quantities from this strain were used to establish a standard curve from Ct values for each primer set. A two-tailed, Student *t*-test was used to identify significant differences between the poultry litter starting material (control) and various incubation conditions.

Autoclaving poultry litter the reduced bacterial genomes (16S rDNA) 1,000-fold and total aerobic plate counts from 10^8 CFU/g (control) to below the limits of detec-

tion (10^2 CFU/g). This was also reflected by a significant reduction in DNA obtained from the autoclaved poultry litter compared to the starting material or various incubation conditions (Figure S1). Normalizing gene abundance against total 16S rDNA revealed the different trends the incubation conditions had on *intI1*, *aadA1* and *sul1* gene copy numbers. This normalization removed the impact of biomass size on total gene abundance. Forced aeration of poultry litter increased the ratio of *intI1* and *aadA1* to 16S rDNA by 0.5 to 1.0 Log_{10} ($p < 0.01$) (Figure 3). Only static incubation reduced *sul1*/16S and *intI1*/16S by 0.5 Log_{10} ($p < 0.01$) in poultry litter. As these observations might reflect changes in the microbial community composition of litter following various incubation conditions, 16S rDNA community analysis was used to assess litter community dynamics following these poultry litter treatments.

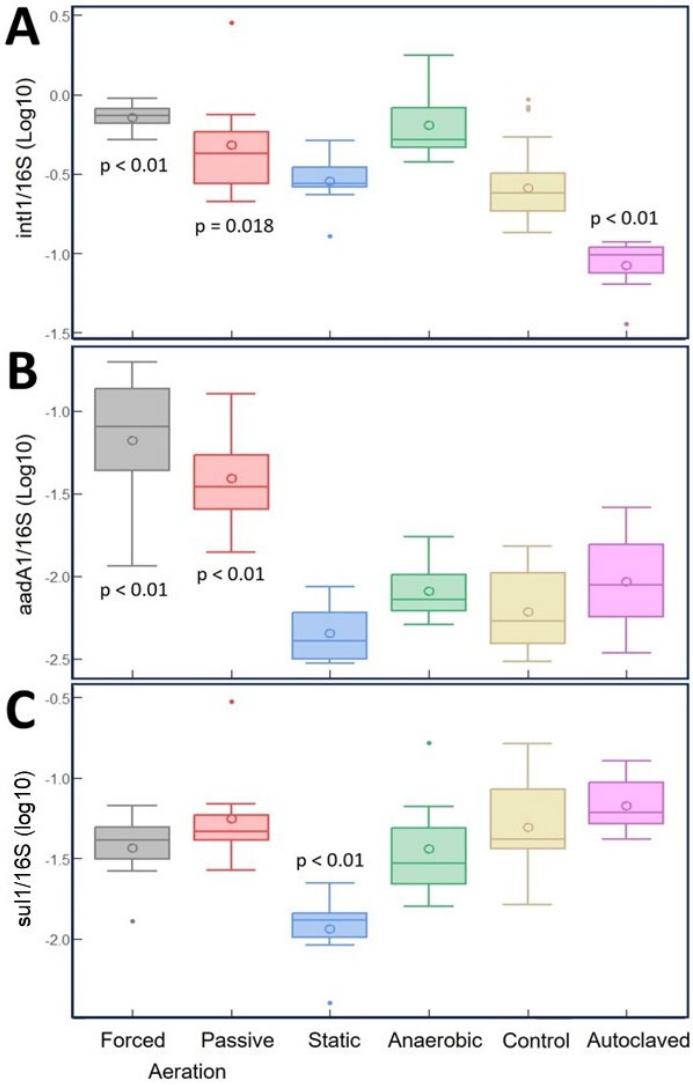

Figure 3. Antimicrobial resistance gene abundance normalized to total bacterial genomes (16S rRNA) in poultry litter before (control) and after various incubation conditions. qPCR was used to quantify genome (16S rRNA) and gene (*intI1*, *aadA1* and *sul1*) abundance. (**A**) *intI1*/16S (Log 10). (**B**) *aadA1*/16S (Log10). (**C**) *sul1*/16S (Log 10). DNA quantities were normalized to 10 ng per PCR reaction. *Escherichia coli* with pDU202 served as positive controls and known DNA quantities from this strain were used to establish a standard curve from Ct values for each primer set. A two-tailed, Student *t*-test was used to identify significant differences between the poultry litter starting material (control) and various incubation conditions.

3.2. Changes in Class 1 Integron-Associated Antibiotic Resistance Genes aadA1 and sul1 Reflect Alterations in Bacterial Community Composition in Response to Litter Incubation Conditions

A culture-independent 16S rDNA metagenomic approach was used to characterize the microbial community composition following various poultry litter incubation conditions. Incubation conditions significantly altered the alpha and beta diversity of the poultry litter bacteriome. Passive and forced aeration increased the community α-diversity ($p < 0.01$) of the litter bacteriome, while static incubation decreased it (Table 2). There was an inverse correlation between Shannon diversity and sul1 or intI1 gene abundance in litter following passive aeration ($p < 0.05$).

Table 2. Impact of incubation conditions on poultry litter community 16S rDNA α-diversity.

Conditions	Reciprocal Simpson		Chao		Shannon		Observed Species	
	Average [1]	*p*-Value [2]	Average [1]	*p*-Value [2]	Average [1]	*p*-Value [2]	Average [1]	*p*-Value [2]
Control (*n* = 32)	20.38 ± 0.74		131.20 ± 3.78		5.33 ± 0.06		129.38 ± 3.67	
Aeration								
Passive (*n* = 8)	32.33 ± 2.75	0.0015	162.26 ± 7.67	0.0021	5.98 ± 0.08 [3]	<0.0001	160.77 ± 7.48	0.0016
Forced (*n* = 8)	31.70 ± 2.49	0.0011	184.39 ± 6.59	<0.0001	6.01 ± 0.07	<0.0001	181.43 ± 6.24	<0.0001
Anaerobic (*n* = 8)	20.31 ± 0.79	0.4749	140.56 ± 4.83	0.0729	5.37 ± 0.05	0.2769	138.41 ± 4.67	0.0735
Static (*n* = 8)	15.28 ± 1.35	0.0035	96.80 ± 2.18	<0.0001	4.87 ± 0.09	0.0004	95.95 ± 2.14	<0.0001

[1] ±SEM. [2] Paired Student *t*-test comparison to poultry litter starting material (control). [3] Pearson correlation coefficient r = −0.7159 ($p = 0.0181$) and −0.7397 ($p < 0.0137$) for *sul1* and *intI1*, respectively.

Regardless of incubation conditions, the Gram-positives Actinobacteria and Firmicutes were the dominant bacterial phyla (Figure 4) in the litter. However, there was an increase of 12 and 18%, in Proteobacteria under forced and passive aeration, respectively, compared to 0% for all other incubation conditions including the control, starting material. The abundance of Actinobacteria varied across incubation conditions, with an observed decrease in abundance relative to that of Firmicutes under anerobic incubation or that of emergent Proteobacteria under passive and forced aeration.

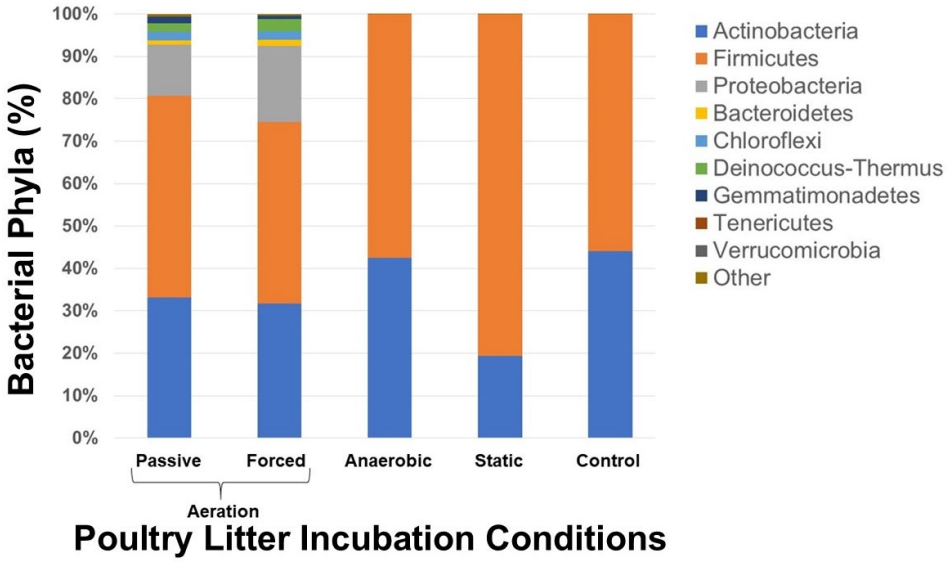

Figure 4. The 16S rRNA metagenome analysis at the phyla level of the poultry litter bacteriome following various incubation conditions. Poultry litter as a starting material served as the control. Poultry litter was rehydrated to 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days.

Changes in the bacterial community were especially evident in the community β-diversity for the litter bacteriome following the various incubation conditions relative to the

starting material (Figure 5) or community comparisons for the different incubation conditions (Figure 6). There was a clear delineation in community composition following passive and forced aeration compared to the control starting material, while there was community overlap with these two incubation conditions compared to anaerobic and static treatments. A substantial change in the poultry litter microbiota was evident at the family level for all incubation conditions (Figure 7). All incubation conditions reduced or eliminated bacteria belonging to *Peptostreptococcaceae*, Clostridiales Family XI, *Clostridiaceae*, *Lactobacillaceae*, *Nocardioidaceae*, and *Dietziaceae*, while promoting the growth of *Micrococcaceae*, *Bacillaceae*, and *Carnobacteriaceae*. Passive and forced aeration amplified the abundance of α-proteobacteria *Rhodospirillaceae*, γ-proteobacteria *Halonmonadaceae*, and the Micrococcales *Borgiellaceae* in poultry litter. Following anaerobic incubation, 14% of bacteria in poultry litter belonged to families in the Class Clostridia: *Ruminococcaceae*, *Peptostreptococcaceae*, Clostridiales Family XI, *Caldicoprobacteraceae*, and Thermoanaerobacterales Family III.

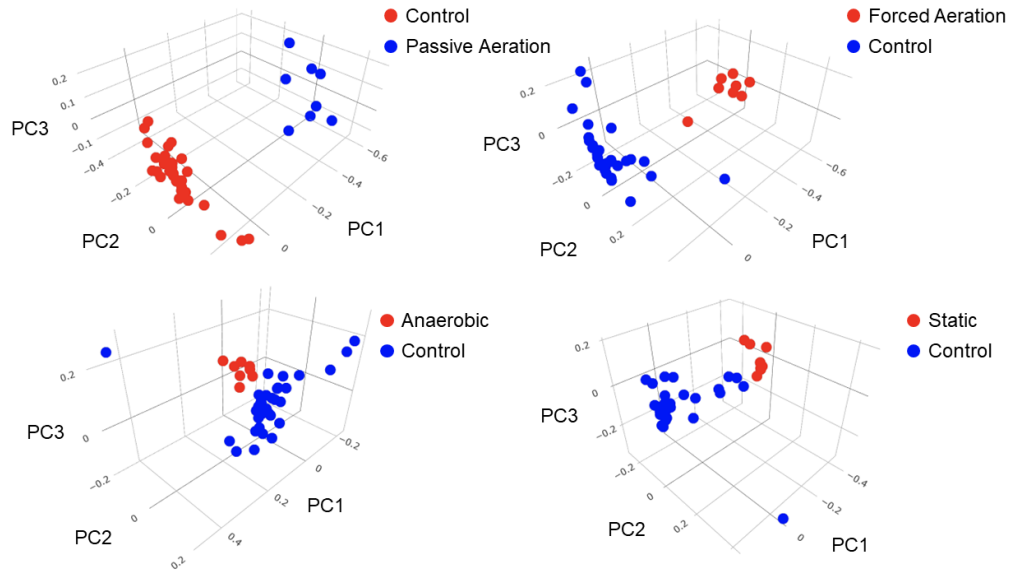

Figure 5. β-diversity of poultry litter bacteriome following various incubation conditions. Poultry litter was rehydrated to a 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days. The starting poultry litter material was untreated. A three-dimensional principal coordinate analysis plot was created using a matrix of paired-wise distance between samples calculated by Bray–Curtis dissimilarity using unique amplicon sequence variants (ASVs).

There was a significant negative correlation ($p < 0.05$) between certain taxa with class 1 integron (*intI1*) or associated antibiotic resistance genes (ARGs) *sul1* or *aadA1* under passive aeration of poultry litter (Table 3). Specifically, there was a negative correlation between class 1 integron and associated ARGs *sul1* and *aadA1* in the Clostridia *Peptococcaceae* and Clostridiales not assigned to a known family under passive aeration. ARG *sul1* had a negative correlation with the taxon *Mycobacterium*, and taxa at the species level, including, *Nocardiopsis alba-algeriensis*, *Nocardiopsis nikkonensis*, *Ammoniphilus oxalaticus*, *Caldicoprobacter* sp30143, *Desulfotomaculum*, *Fodinicurvata sediminis*, *Achromobacter* sp48100, and an unknown *Cytophagia*; it also had a negative correlation between *aadA1* and an unknown *Rhodothermaceae* and unknown *Sphaerobacteraceae*, and a negative correlation between *sul1* and *intI1* with taxa belonging to *Saccharomonospora*, *Nocardiopsis*, and *Nocardiopsis* under passive aeration. There were fewer correlations between *intI1*, *sul1* or *aadA1* with taxa at the species level under static (*Bacillaceae* sp26447) or anaerobic (*Atopostipes* sp28157, *Anaerosalibacter* sp31230-sp31231) conditions. There was no correlation in abundance between any taxa with *intI1*, *sul1* or *aadA1* with the control starting litter material.

In addition, no significant positive correlations were observed between bacterial taxa and intI1 or associated ARGs *sul1* or *aadA1* under any incubation conditions.

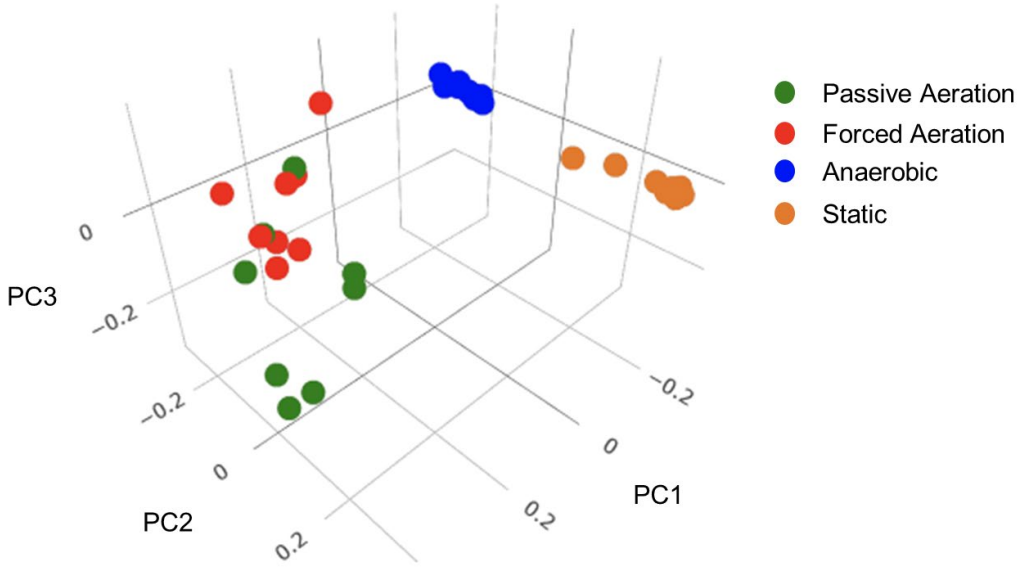

Figure 6. Comparison of bacterial community β-diversity of poultry litter following the aerobic, static, anaerobic, or periodic wetting (wet-dry) of poultry litter. Poultry litter was rehydrated to a 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days. A three-dimensional principal coordinate analysis plot was created using a matrix of paired-wise distance between samples calculated by Bray–Curtis dissimilarity using unique amplicon sequence variants (ASVs).

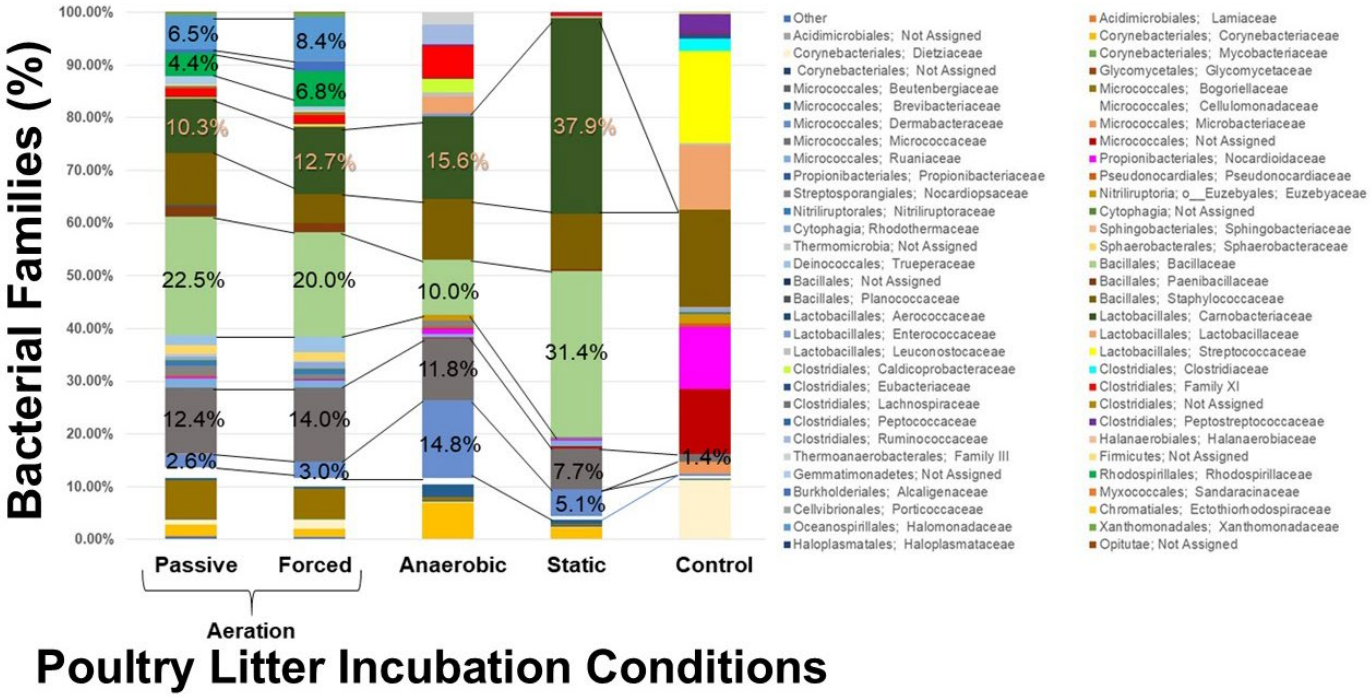

Figure 7. The 16S metagenome analysis at the genus level of the poultry litter microbiota following various incubation conditions. The poultry litter, as the starting material, served as the control. Poultry litter was rehydrated to 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days.

Table 3. Correlation between family or genera with class 1 integron and associated antimicrobial resistance gene abundance as a function of litter incubation conditions.

Family [1]/Genus [2]/Species	*aadA1*		*sul1*		*intI1*	
	r =	*p* =	r =	*p* =	r =	*p* =
Passive Aeration						
Actinobacteria						
Mycobacteriaceae			−0.74	0.014		
Mycobacterium			−0.74	0.014		
Pseudonocardiaceae			−0.80	0.005	−0.760	0.010
Saccharomonospora			−0.90	<0.001	−0.87	0.001
Nocardiopsaceae			−0.84	0.003	−0.79	0.006
Nocardiopsis			−0.85	0.002	−0.78	0.008
alba−algeriensis			−0.76	0.010		
nikkonensis			−0.76	0.010		
Firmicutes						
Paenibacillaceae						
Ammoniphilus			−0.80	0.005	−0.81	0.005
oxalaticus			−0.80	0.005		
Caldicoprobacteraceae						
*Caldicoprobacter sp*30143			−0.72	0.017		
Clostridiales; Family XI						
Tepidimicrobium			−0.73	0.016		
*Tissierella sp*31385−sp31390			−0.76	0.010		
Clostridiales; Not Assigned	−0.72	0.018	−0.77	0.010	−0.72	0.018
Peptococcaceae	−0.81	0.005	−0.83	0.003	−0.74	0.013
Desulfotomaculum			−0.73	0.016		
Proteobacteria						
Rhodospirillaceae	−0.77	0.009	−0.79	0.007	−0.75	0.012
Fodinicurvata			−0.79	0.0065	−0.73	0.016
sediminis			−0.79	0.006		
Alcaligenaceae			−0.70	0.020		
Achromobacter			−0.76	0.011		
*sp*48100			−0.76	0.011		
Bacteroidetes			−0.73	0.015		
Cytophagia; Not Assigned			−0.71	0.019		
Rhodothermaceae	−0.74	0.021				
Chlorofexi						
Sphaerobacteraceae	−0.70	0.021				
Static						
Firmicutes						
Bacillaceae						
*sp*26447			−0.76	0.011	−0.76	0.001
Anaerobic						
Firmicutes						
Bacillaceae						
*Atopostipes sp*28157	−0.78	<0.001				
*Anaerosalibacter sp*31230−*sp*31231	−0.74	0.002				

[1] Families (n = 61). [2] Genera (n = 149).

3.3. Poultry Litter Maintains Its Nutrient Concentration as a Soil Amendment Under Various Incubation Conditions

As expected, the greater nitrogen and lower C:N ratio of the litter following incubation resulted in a reduced C/N ratio close to 11:1. Typically, the C/N ratio during decomposition of organics approaches a value of around 10:1 in completely aged compost, a value typical of C/N in soils [68,69]. The values of C/N observed of approximately 11:1 are comparable to the values cited in the literature and indicate that the decomposition process went as expected (Figure 8). The lower C contents with forced and passive aeration, held at 45 °C, were expected, compared to those in the static condition, which was maintained at room temperature. At 45 °C, decomposition rates were higher and reflect what happens inside piles of poultry litter relative to ambient conditions at the surface. At a depth of 15 cm or greater, internal temperatures can rise to higher values within a poultry litter pile because of the self-insulating effect. Losses of nitrogen were the lowest in the anaerobic treatment,

compared to the other incubation conditions, which typically underwent a drop in pH due to the accumulation of short chain fatty acids, intermediates in the anaerobic breakdown process. The lower pH reduced the volatilization of nitrogen in the form of ammonia. It is important to note that even under high forced aeration conditions, the nitrogen content of the poultry litter at the end of 28 d was approximately 2.75%. Such aerobically stabilized poultry litter would therefore continue to have value as a nitrogen-source soil amendment.

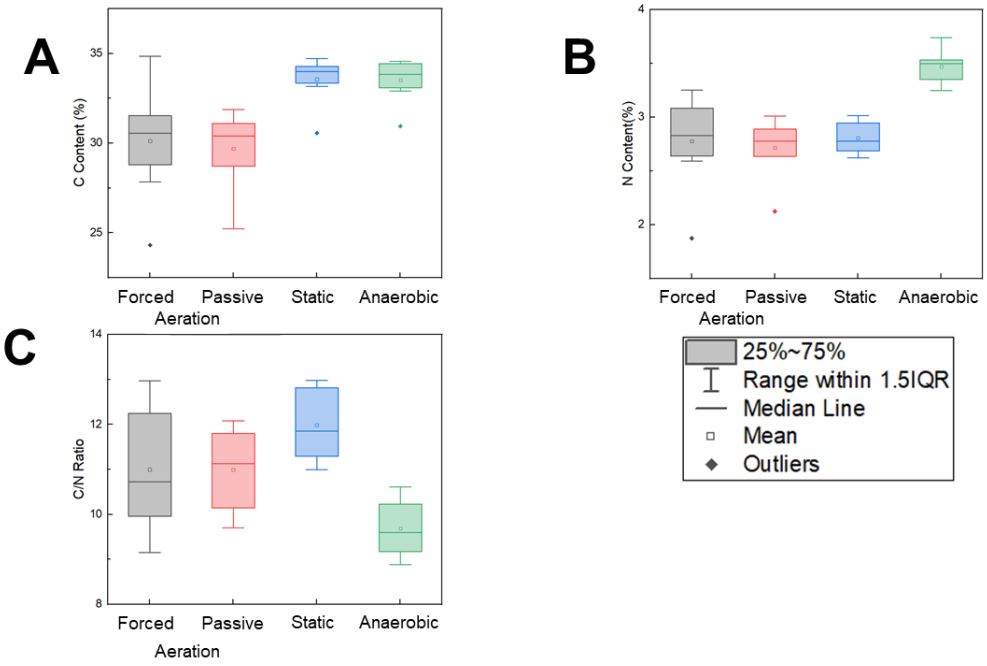

Figure 8. Nutrient concentration of poultry litter following different incubation conditions. (**A**) Carbon content. (**B**) Nitrogen content. (**C**) Carbon/nitrogen ratio. Poultry litter was rehydrated to a 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days.

4. Discussion

Class 1 integrons capture antimicrobial resistance genes (ARGs), building resistance upon resistance within its integration site [19]. This genetic element has been reported in both Gram-negatives and Gram-positives [19], especially in pathogens that inhabit the poultry environment [1,20,70]. In poultry litter, ~11% of the litter microbiota or 10^7 bacteria/g poultry litter possess class 1 integron [1]. As an amendment, poultry litter introduces a sizable gene load of class 1 integron and associated ARG into soil. Class 1 integron has been reported in bacteria isolated from various environments including soil [19]. Can poultry litter be remediated of class 1 integrons and its associated ARG, while retaining its nutrient concentration as a soil amendment for crop growth?

Different poultry litter incubation conditions were examined to identify a litter treatment that could significantly reduce the abundance of class 1 integron and associated ARGs sul1 and aadA1. Several incubation conditions were identified that either increased (passive and forced aeration) or decreased (static, anaerobic) the abundance of class 1 integron marker *intI1* or ARGs *sul1* or *aadA1*, commonly associated with integrons, relative to the control starting material. With the exception of autoclaving, ARG abundances declined by 0.5 to 1.0 Log_{10} at most, and it thus unclear whether a 50 to 90% reduction in the large pool of ARGs is sufficient to alter risk. Others have also reported minor changes in class 1 integron and associated ARGs with the following treatments: windrowing and litter amendment with aluminosilicate [71]; passive aeration with a surfactant amendment [54]; and composting with daily turning of compost [53]. Anaerobic digestions of cattle, dairy, or swine manure have been reported to affect class 1 integron and associated ARGs aadA1 and

sul1, although only reducing their abundances by 0.5–1.0 Log_{10} copies/g manure [72–74]. The results support those other reports but also extend their lines of research by addressing the impact of five different incubation conditions on the ARG loads and bacteriome and nutrient concentrations of composted poultry litter as a soil amendment. These changes in ARG loads were less pronounced when normalized against total bacterial genomes in poultry litter. The significant differences that were observed in ARG per 16S rDNA copies reflected changes in the bacterial composition and the correlation between ARG abundance with certain bacterial taxa.

The different litter processes changed the microbiome substantially. Microbial succession occurs as poultry litter matures, starting with fresh bedding, where Gram-Negatives, α-, γ-proteobacteria and Bacteroidia are the dominant phyla to be later replaced by the Gram-positive phyla Actinobacteria and Firmicutes [30,75]. Several different litter processes have been reported to profoundly affect litter microbiome composition. Subirats et al. reported a decline in the Gram-positives Actinobacteria and Firmicutes with a corresponding increase in the abundance of the Gram-negatives Bacteroidetes and Proteobacteria under the composting (passive aeration; 27 °C; amended with sawdust and hay) of raw litter [53]. Microbial succession was reported in Gram-positives under windrowing, starting with Clostridiales Family XI as the most abundant group, followed by *Bacillaceae* (day 15), supplanted by *Staphylococcaceae* (day 28), and finally *Paenibacillaceae* (day 39) [71]. On the other hand, Clostridia populations increased following the anaerobic digestion of poultry litter [76]. Similarly, changes in bacterial populations were observed with the different litter incubation conditions described in this study. Aeration amplified the Proteobacteria population in poultry litter while reducing that of anaerobic Clostridia. However, anaerobic digestion augmented Clostridia including Clostridiales Family XI. These changes in community composition may account for the reduction in class 1 integron and its associated ARG, however slight. Others have reported similar correlations between taxa and ARG abundance [71,77–80]. A negative correlation was observed between the abundance of certain taxa and class 1 integrons or associated ARGs with passive, static or anaerobic incubations. Passive aeration had the most pronounced effect on 26 bacterial taxa and the abundance of class 1 integrons or their associated ARGs *aadA1* and *sul1*. Negative correlations observed for genera *Caldicoprobacter* and *Saccharomonospora* with the integron and integron-associated ARGs in this study have been reported in others involving composting or the anaerobic digestion of litter [41,78,79]. However, there were no positive correlations between taxa and the class 1 integrons or associated ARGs with any of the incubation conditions examined in this study.

While the different incubation conditions may not have affected class 1 integron abundance in poultry litter, that of the ARGs within *attI1* integration may have changed with the alteration in the microbial community composition. In addition to *aadA1*, other *aadA* alleles and trimethoprim resistance gene *dfrA1* are present in *attI1* site of *Corynebacterium* species isolated from poultry litter [1]. Aminoglycoside and chloramphenicol resistance genes have been identified in the class 1 integron resistome of poultry litter [13]. High-throughput sequencing of class 1 integron cassette arrays [81] in poultry litter treatments might identify an incubation condition that significantly alters the class 1 integron resistome.

The inability to substantially reduce the abundance of class 1 integron and its associated ARGs, by more than one Log10, may be attributed to their broad dissemination of diverse phyla including Proteobacteria, Firmicutes and Actinobacteria [13]. Only autoclaving significantly reduced ARG abundance by 3 Log_{10}, mainly because it killed most of the bacterial population. Steam explosion (autoclaving) has been explored to improve the bioavailability of plant and bacterial cell wall material in animal feed [82]. It has the added value of also eliminating pathogens from animal wastes. The challenge will be in

scaling up this process to an industrial scale for processing tons of animal waste. Except for autoclaving, none of the litter processes markedly reduced ARG abundance. However, ε-proteobacteria, *Enterobacteriaceae* and *Streptococcaceae*, which include pathogenic genera like *Escherichia*, *Salmonella*, *Campylobacter* and *Enterococcus*, were not detected in any of 16S rRNA sequences following poultry litter incubation under aerobic or anaerobic conditions. None of the incubation conditions had a significant impact on *Staphylococcaceae* abundance. While *Staphylococcaceae* can be abundant in poultry litter [13,83], they generally do not exhibit the broad antimicrobial resistance observed in *Enterococcus* [83]. Reducing zoonotic pathogens like Salmonella on the poultry product, in turn, reduces the abundance of any antibiotic-resistant variant as well [84]. Anaerobic digestion may have the added benefit of augmenting anaerobic symbionts for seeding young birds and promoting healthy gut development [85,86]. Finally, none of the incubations affected the nutrient concentration of poultry litter as a soil amendment.

5. Conclusions

As a soil amendment, animal manure is a valuable commodity for organic farming. Unfortunately, it contains zoonotic pathogens and a large and diverse ARG reservoir. Reducing occurrence would be a first line for offsetting ARG transmission to animals and humans and maintaining the treatment efficacy of current antimicrobials. This study assessed different poultry litter incubation conditions at reducing antimicrobial resistances associated with class 1 integrons. It was expected that stressful environmental conditions, those that vary from those for ideal bacterial growth conditions, would alter integron load, and promote or deter cassette transmission. Except for autoclaving, the reduction in *aadA1* and sul1 abundance was minimal (0.5–1.0 Log_{10}/g decrease) under the conditions investigated. However, nominal differences in antimicrobial resistance gene load appeared tied to changes in the poultry litter microbiota. As class 1 integrons and associated ARGs *aadA1* and *sul1* are broadly distributed across diverse bacterial taxa in poultry litter, no one incubation condition is expected to substantially reduce this ARG load. Similarly, specific AMR phenotypes, for example tetracycline resistance, are encoded by a diverse array of antimicrobial resistance genes or alleles across many bacterial taxa. Only processes that dramatically lower the total bacterial population, like steam explosion (autoclaving), are expected to substantially reduce ARG load in animal manures. It is not clear if the small reductions in ARG load would result in biologically meaningful reductions in the risk of AMR transmission. However, class 1 integrons and associated ARG in the poultry litter do not appear to be linked to conjugative plasmids that could spread associated resistances. If any one of these poultry litter processes can eradicate zoonotic pathogens, then it would also be able to eliminate antimicrobial resistances associated with said pathogens.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture15040398/s1: Figure S1. Total DNA per gram of poultry litter and processed poultry litter. Poultry litter starting material served as the control. Poultry litter was rehydrated to 55% water content and incubated at 45 °C (passive aeration, forced aeration, anaerobic) or 25 °C (static) for 28 days. Bacterial pellets were extracted from one-gram samples. DNA was extracted from bacterial pellets with ZymoBiomics DNA Miniprep Kit and quantified using NanoDrop One. Control was poultry litter, pre-treatment. * Significant difference as determined by paired, two-tailed student *t*-test between treatment and control ($p < 0.01$). Autoclaved litter had significantly lower DNA yields (state reduction, log10 relative to control or compare DNA amounts + SEM for control and autoclaved samples) compared to the control ($p < 0.01$).

Author Contributions: Conceptualization, K.C.D. and J.J.M.; methodology, A.H., J.W., M.D.L. and M.A.W.; software, A.H., S.K. and J.J.M.; validation, J.J.M., M.D.L., M.A.W. and R.B.; formal analysis, A.H., J.J.M., S.K. and R.B.; investigation, G.P.P., K.C.D. and J.W.; resources, G.P.P. and K.C.D.; data

curation, A.H. and J.J.M.; writing—original draft preparation, J.J.M.; writing—review and editing, J.J.M., A.H., K.C.D., M.A.W., M.D.L., C.R. and R.B.; visualization, A.H., J.W. and J.J.M.; supervision, K.C.D. and J.J.M.; project administration, J.J.M.; funding acquisition, J.J.M., K.C.D., M.A.W., C.R. and R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the United States Department of Agriculture (2017-05586; VA-160130).

Institutional Review Board Statement: This study did not involve animals or humans. The research described in this publication was approved by Virginia Polytechnic Institute and State University Institutional Biosafety Committee 22-035 "Using a microbiome approach to reducing resistome in poultry litter amended soils".

Data Availability Statement: The data presented in this study are openly available in the National Center for Biotechnology Information under Bioproject accession PRJNA1082344 at [NCBI] [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1082344] (accessed on 4 March 2024).

Conflicts of Interest: The authors declare there were no commercial or financial relationships associated with any of the research described in this publication.

References

1. Nandi, S.; Maurer, J.J.; Hofacre, C.; Summers, A.O. Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7118–7122. [CrossRef]
2. King, G.M.; Brooks, J.P.; Brown, S.; Gerba, C.; O'Connor, G.A.; Pepper, I.L. *Land Application of Organic Residuals: Public Health Threat or Environmental Benefit*; American Society for Microbiology: Washington, DC, USA, 2011.
3. Hutchison, M.L.; Walters, L.D.; Avery, S.M.; Munro, F.; Moore, A. Analyses of livestock production, waste storage, and pathogen levels and prevalences in farm manures. *Appl. Environ. Microbiol.* **2005**, *71*, 1231–1236. [CrossRef]
4. Pornsukarom, S.; Thakur, S. Horizontal Dissemination of Antimicrobial Resistance Determinants in Multiple Salmonella Serotypes following Isolation from the Commercial Swine Operation Environment after Manure Application. *Appl. Environ. Microbiol.* **2017**, *83*, e01503-17. [CrossRef]
5. Bennett, S.D.; Sodha, S.V.; Ayers, T.L.; Lynch, M.F.; Gould, L.H.; Tauxe, R.V. Produce-associated foodborne disease outbreaks, USA, 1998–2013. *Epidemiol Infect* **2018**, *146*, 1397–1406. [CrossRef]
6. Carstens, C.K.; Salazar, J.K.; Darkoh, C. Multistate Outbreaks of Foodborne Illness in the United States Associated With Fresh Produce From 2010 to 2017. *Front. Microbiol.* **2019**, *10*, 2667. [CrossRef]
7. Sivapalasingam, S.; Friedman, C.R.; Cohen, L.; Tauxe, R.V. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J. Food Prot.* **2004**, *67*, 2342–2353. [CrossRef]
8. Luna, S.; Krishnasamy, V.; Saw, L.; Smith, L.; Wagner, J.; Weigand, J.; Tewell, M.; Kellis, M.; Penev, R.; McCullough, L.; et al. Outbreak of *E. coli* O157:H7 Infections Associated with Exposure to Animal Manure in a Rural Community—Arizona and Utah, June–July 2017. *MMWR Morb. Mortal. Wkly Rep.* **2018**, *67*, 659–662. [CrossRef]
9. Bottichio, L.; Keaton, A.; Thomas, D.; Fulton, T.; Tiffany, A.; Frick, A.; Mattioli, M.; Kahler, A.; Murphy, J.; Otto, M.; et al. Shiga Toxin-Producing Escherichia coli Infections Associated with Romaine Lettuce-United States, 2018. *Clin. Infect Dis.* **2020**, *71*, e323–e330. [CrossRef]
10. Soderstrom, A.; Osterberg, P.; Lindqvist, A.; Jonsson, B.; Lindberg, A.; Blide Ulander, S.; Welinder-Olsson, C.; Lofdahl, S.; Kaijser, B.; De Jong, B.; et al. A large Escherichia coli O157 outbreak in Sweden associated with locally produced lettuce. *Foodborne Pathog. Dis.* **2008**, *5*, 339–349. [CrossRef]
11. Zhang, H.; Schroder, J. Animal Manure Production and Utilization in the US. In *Applied Manure and Nutrient Chemistry for Sustainable Agriculture and Environment*; Springer Netherlands: Dordrecht, The Netherlands, 2014; pp. 1–21. [CrossRef]
12. Boesch, D.F.; Brinsfield, R.B.; Magnien, R.E. Chesapeake Bay eutrophication: Scientific understanding, ecosystem restoration, and challenges for agriculture. *J. Environ. Qual.* **2001**, *30*, 303–320. [CrossRef]
13. Lu, J.; Sanchez, S.; Hofacre, C.; Maurer, J.J.; Harmon, B.G.; Lee, M.D. Evaluation of broiler litter with reference to the microbial composition as assessed by using 16S rRNA and functional gene markers. *Appl. Environ. Microbiol.* **2003**, *69*, 901–908. [CrossRef] [PubMed]
14. Zalewska, M.; Błażejewska, A.; Czapko, A.; Popowska, M. Antibiotics and Antibiotic Resistance Genes in Animal Manure—Consequences of Its Application in Agriculture. *Front. Microbiol.* **2021**, *12*, 610656. [CrossRef] [PubMed]

15. He, L.-Y.; Liu, Y.-S.; Su, H.-C.; Zhao, J.-L.; Liu, S.-S.; Chen, J.; Liu, W.-R.; Ying, G.-G. Dissemination of Antibiotic Resistance Genes in Representative Broiler Feedlots Environments: Identification of Indicator ARGs and Correlations with Environmental Variables. *Environ. Sci. Technol.* **2014**, *48*, 13120–13129. [CrossRef] [PubMed]
16. Gillings, M.R. Class 1 integrons as invasive species. *Curr. Opin. Microbiol.* **2017**, *38*, 10–15. [CrossRef]
17. Deng, Y.; Bao, X.; Ji, L.; Chen, L.; Liu, J.; Miao, J.; Chen, D.; Bian, H.; Li, Y.; Yu, G. Resistance integrons: Class 1, 2 and 3 integrons. *Ann. Clin. Microbiol. Antimicrob.* **2015**, *14*, 45. [CrossRef]
18. Hipólito, A.; García-Pastor, L.; Vergara, E.; Jové, T.; Escudero, J.A. Profile and resistance levels of 136 integron resistance genes. *npj Antimicrob. Resist.* **2023**, *1*, 13. [CrossRef]
19. Ghaly, T.M.; Gillings, M.R.; Penesyan, A.; Qi, Q.; Rajabal, V.; Tetu, S.G. The Natural History of Integrons. *Microorganisms* **2021**, *9*, 2212. [CrossRef]
20. Bass, L.; Liebert, C.A.; Lee, M.D.; Summers, A.O.; White, D.G.; Thayer, S.G.; Maurer, J.J. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob. Agents Chemother.* **1999**, *43*, 2925–2929. [CrossRef]
21. Meyers, M.A.; Durso, L.M.; Gilley, J.E.; Waldrip, H.M.; Castleberry, L.; Millmier-Schmidt, A. Antibiotic resistance gene profile changes in cropland soil after manure application and rainfall. *J. Environ. Qual.* **2020**, *49*, 754–761. [CrossRef]
22. Lopatto, E.; Choi, J.; Colina, A.; Ma, L.; Howe, A.; Hinsa-Leasure, S. Characterizing the soil microbiome and quantifying antibiotic resistance gene dynamics in agricultural soil following swine CAFO manure application. *PLoS ONE* **2019**, *14*, e0220770. [CrossRef]
23. Ruuskanen, M.; Muurinen, J.; Meierjohan, A.; Parnanen, K.; Tamminen, M.; Lyra, C.; Kronberg, L.; Virta, M. Fertilizing with Animal Manure Disseminates Antibiotic Resistance Genes to the Farm Environment. *J. Environ. Qual.* **2016**, *45*, 488–493. [CrossRef] [PubMed]
24. Guron, G.K.P.; Arango-Argoty, G.; Zhang, L.; Pruden, A.; Ponder, M.A. Effects of Dairy Manure-Based Amendments and Soil Texture on Lettuce- and Radish-Associated Microbiota and Resistomes. *mSphere* **2019**, *4*, 1–14. [CrossRef] [PubMed]
25. Zhang, Y.J.; Hu, H.W.; Chen, Q.L.; Singh, B.K.; Yan, H.; Chen, D.; He, J.Z. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. *Environ. Int.* **2019**, *130*, 104912. [CrossRef] [PubMed]
26. Zhang, Y.J.; Hu, H.W.; Chen, Q.L.; Yan, H.; Wang, J.T.; Chen, D.; He, J.Z. Manure Application Did Not Enrich Antibiotic Resistance Genes in Root Endophytic Bacterial Microbiota of Cherry Radish Plants. *Appl. Environ. Microbiol.* **2020**, *86*, e02106-19. [CrossRef]
27. Henuk, Y.L.; Dingle, J.G. Poultry manure: Source of fertilizer, fuel and feed. *World's Poult. Sci. J.* **2003**, *59*, 350–360. [CrossRef]
28. Ashworth, A.J.; Chastain, J.P.; Moore, P.A. Nutrient characteristics of poultry manure and litter. In *Animal Manure: Production, Characteristics, Environmental Concerns, and Management*; Waldrip, H.M., Pagliari, P.H., He, Z., Eds.; Wiley Online Library: Hoboken, NJ, USA, 2020; pp. 63–87.
29. Vieira, S.; Moran, E., Jr. Effects of delayed placement and used litter on broiler yields. *J. Appl. Poultry Res.* **1999**, *8*, 75–81. [CrossRef]
30. Cressman, M.D.; Yu, Z.; Nelson, M.C.; Moeller, S.J.; Lilburn, M.S.; Zerby, H.N. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Appl. Environ. Microbiol.* **2010**, *76*, 6572–6582. [CrossRef]
31. Hill, D.; Morra, M.J.; Stalder, T.; Jechalke, S.; Top, E.; Pollard, A.T.; Popova, I. Dairy manure as a potential source of crop nutrients and environmental contaminants. *J. Environ. Sci.* **2021**, *100*, 117–130. [CrossRef]
32. Kumar, A.; Gupta, D.K.; Kumar, M. Green manure crops: A boon for agricultural soil. *Int. J. Agric. Environ. Biotechnol.* **2013**, *6*, 193–198.
33. Fussell, L.W. Poultry industry strategies for control of immunosuppressive diseases. *Poult. Sci.* **1998**, *77*, 1193–1196. [CrossRef]
34. Gurtler, J.B.; Doyle, M.P.; Erickson, M.C.; Jiang, X.; Millner, P.; Sharma, M. Composting To Inactivate Foodborne Pathogens for Crop Soil Application: A Review. *J. Food Prot.* **2018**, *81*, 1821–1837. [CrossRef] [PubMed]
35. Arikan, O.A.; Sikora, L.J.; Mulbry, W.; Khan, S.U.; Foster, G.D. Composting rapidly reduces levels of extractable oxytetracycline in manure from therapeutically treated beef calves. *Bioresour. Technol.* **2007**, *98*, 169–176. [CrossRef] [PubMed]
36. Arikan, O.; Mulbry, W.; Ingram, D.; Millner, P. Minimally managed composting of beef manure at the pilot scale: Effect of manure pile construction on pile temperature profiles and on the fate of oxytetracycline and chlortetracycline. *Bioresour. Technol.* **2009**, *100*, 4447–4453. [CrossRef] [PubMed]
37. Sharma, R.; Larney, F.J.; Chen, J.; Yanke, L.J.; Morrison, M.; Topp, E.; McAllister, T.A.; Yu, Z. Selected antimicrobial resistance during composting of manure from cattle administered sub-therapeutic antimicrobials. *J. Environ. Qual.* **2009**, *38*, 567–575. [CrossRef]
38. Kim, K.R.; Owens, G.; Ok, Y.S.; Park, W.K.; Lee, D.B.; Kwon, S.I. Decline in extractable antibiotics in manure-based composts during composting. *Waste Manag.* **2012**, *32*, 110–116. [CrossRef]
39. Qian, X.; Sun, W.; Gu, J.; Wang, X.J.; Sun, J.J.; Yin, Y.N.; Duan, M.L. Variable effects of oxytetracycline on antibiotic resistance gene abundance and the bacterial community during aerobic composting of cow manure. *J. Hazard. Mater.* **2016**, *315*, 61–69. [CrossRef]
40. Dolliver, H.; Gupta, S.; Noll, S. Antibiotic degradation during manure composting. *J. Environ. Qual.* **2008**, *37*, 1245–1253. [CrossRef]

41. Peng, S.; Wang, Y.; Zhou, B.; Lin, X. Long-term application of fresh and composted manure increase tetracycline resistance in the arable soil of eastern China. *Sci. Total Environ.* **2015**, *506–507*, 279–286. [CrossRef]
42. Guan, J.; Wasty, A.; Grenier, C.; Chan, M. Influence of temperature on survival and conjugative transfer of multiple antibiotic-resistant plasmids in chicken manure and compost microcosms. *Poult. Sci.* **2007**, *86*, 610–613. [CrossRef]
43. Cui, E.; Wu, Y.; Zuo, Y.; Chen, H. Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. *Bioresour. Technol.* **2016**, *203*, 11–17. [CrossRef]
44. Erickson, M.C.; Smith, C.; Jiang, X.; Flitcroft, I.D.; Doyle, M.P. Manure source and age affect survival of zoonotic pathogens during aerobic composting at sublethal temperatures. *J. Food Prot.* **2015**, *78*, 302–310. [CrossRef] [PubMed]
45. Erickson, M.C.; Smith, C.; Jiang, X.; Flitcroft, I.D.; Doyle, M.P. Survival of Salmonella or Escherichia coli O157:H7 during holding of manure-based compost mixtures at sublethal temperatures as influenced by the carbon amendment. *J. Food Prot.* **2015**, *78*, 248–255. [CrossRef] [PubMed]
46. Arikan, O.A.; Sikora, L.J.; Mulbry, W.; Khan, S.U.; Rice, C.; Foster, G.D. The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. *Process Biochem.* **2006**, *41*, 1637–1643. [CrossRef]
47. Ghosh, S.; Ramsden, S.J.; LaPara, T.M. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **2009**, *84*, 791–796. [CrossRef]
48. Zhang, T.; Yang, Y.; Pruden, A. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7771–7779. [CrossRef]
49. Byrne-Bailey, K.G.; Gaze, W.H.; Zhang, L.; Kay, P.; Boxall, A.; Hawkey, P.M.; Wellington, E.M. Integron prevalence and diversity in manured soil. *Appl. Environ. Microbiol.* **2011**, *77*, 684–687. [CrossRef]
50. You, Y.; Hilpert, M.; Ward, M.J. Identification of Tet45, a tetracycline efflux pump, from a poultry-litter-exposed soil isolate and persistence of tet (45) in the soil. *J. Antimicrob. Chemoth* **2013**, *68*, 1962–1969. [CrossRef]
51. Gillings, M.R.; Gaze, W.H.; Pruden, A.; Smalla, K.; Tiedje, J.M.; Zhu, Y.-G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME journal* **2015**, *9*, 1269–1279. [CrossRef]
52. Marti, R.; Scott, A.; Tien, Y.-C.; Murray, R.; Sabourin, L.; Zhang, Y.; Topp, E. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. *Appl. Environ. Microbiol.* **2013**, *79*, 5701–5709. [CrossRef]
53. Subirats, J.; Murray, R.; Yin, X.; Zhang, T.; Topp, E. Impact of chicken litter pre-application treatment on the abundance, field persistence, and transfer of antibiotic resistant bacteria and antibiotic resistance genes to vegetables. *Sci. Total Environ.* **2021**, *801*, 149718. [CrossRef]
54. Zhang, Y.; Li, H.; Gu, J.; Qian, X.; Yin, Y.; Li, Y.; Zhang, R.; Wang, X. Effects of adding different surfactants on antibiotic resistance genes and intI1 during chicken manure composting. *Bioresour. Technol.* **2016**, *219*, 545–551. [CrossRef] [PubMed]
55. Ritz, C.W.; John, W. Poultry Mortality Composting Management Guide. Available online: https://extension.uga.edu/publications/detail.html?number=B1266&title=poultry-mortality-composting-management-guide (accessed on 24 October 2024).
56. Liang, C.; Das, K.C.; McClendon, R.W. The influence of temperature and moisture contents regimes on the aerobic microbial activity of a biosolids composting blend. *Bioresour. Technol.* **2003**, *86*, 131–137. [CrossRef] [PubMed]
57. Anonymous. *In-Vessel Composting of Municipal Wastewater Sludge*; U.S. Environmental Protection Agency, Center for Environmental Research Information: Washington, DC, USA, 1989.
58. Leege, P.B. Compost Facility Operating Guide. In *The Science of Composting*; de Bertoldi, M., Sequi, P., Lemmes, B., Papi, T., Eds.; Springer Netherlands: Dordrecht, The Netherlands, 1996; pp. 126–136. [CrossRef]
59. Weyers, S.L.; Das, K.C.; Gaskin, J.W.; Liesch, A.M. Pine Chip and Poultry Litter Derived Biochars Affect C and N Dynamics in Two Georgia, USA, Ultisols. *Agronomy* **2023**, *13*, 531. [CrossRef]
60. Anonymous. *Test Methods for the Evaluation of Composting And composted Products*; The U. S. Composting Council: Alexandria, VA, USA, 1997.
61. Oxendine, A.; Walsh, A.A.; Young, T.; Dixon, B.; Hoke, A.; Rogers, E.E.; Lee, M.D.; Maurer, J.J. Conditions Necessary for the Transfer of Antimicrobial Resistance in Poultry Litter. *Antibiotics* **2023**, *12*, 1006. [CrossRef]
62. Provence, D.L.; Curtiss, R., III. *Gene Transfer in Gram-Negative Bacteria*; ASM Press: Washington, DC, USA, 1994.
63. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
64. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **2011**, *17*, 10–12. [CrossRef]
65. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef]
66. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]

67. McDonald, D.; Price, M.N.; Goodrich, J.; Nawrocki, E.P.; DeSantis, T.Z.; Probst, A.; Andersen, G.L.; Knight, R.; Hugenholtz, P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **2012**, *6*, 610–618. [CrossRef]
68. Choi, I.; Moore, P., Jr. Effect of various litter amendments on ammonia volatilization and nitrogen content of poultry litter. *J. Appl. Poultry Res.* **2008**, *17*, 454–462. [CrossRef]
69. Zuberer, D.A.; Zibilske, L.M. Composting: The microbiological processing of organic wastes. In *Principles and Applications of Soil Microbiology*; Elsevier: Amsterdam, The Netherlands, 2021; pp. 655–679.
70. Goldstein, C.; Lee, M.D.; Sanchez, S.; Hudson, C.; Phillips, B.; Register, B.; Grady, M.; Liebert, C.; Summers, A.O.; White, D.G.; et al. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. *Antimicrob. Agents Chemother.* **2001**, *45*, 723–726. [CrossRef] [PubMed]
71. Peng, S.; Li, H.; Song, D.; Lin, X.; Wang, Y. Influence of zeolite and superphosphate as additives on antibiotic resistance genes and bacterial communities during factory-scale chicken manure composting. *Bioresour. Technol.* **2018**, *263*, 393–401. [CrossRef] [PubMed]
72. Tran, T.T.; Scott, A.; Tien, Y.C.; Murray, R.; Boerlin, P.; Pearl, D.L.; Liu, K.; Robertson, J.; Nash, J.H.E.; Topp, E. On-Farm Anaerobic Digestion of Dairy Manure Reduces the Abundance of Antibiotic Resistance-Associated Gene Targets and the Potential for Plasmid Transfer. *Appl. Environ. Microbiol.* **2021**, *87*, e0298020. [CrossRef] [PubMed]
73. Sun, W.; Qian, X.; Gu, J.; Wang, X.J.; Duan, M.L. Mechanism and Effect of Temperature on Variations in Antibiotic Resistance Genes during Anaerobic Digestion of Dairy Manure. *Sci. Rep.* **2016**, *6*, 30237. [CrossRef]
74. Zalewska, M.; Błażejewska, A.; Szadziul, M.; Ciuchciński, K.; Popowska, M. Effect of composting and storage on the microbiome and resistome of cattle manure from a commercial dairy farm in Poland. *Environ. Sci. Pollut. Res. Int.* **2024**, *31*, 30819–30835. [CrossRef]
75. Kubasova, T.; Kollarcikova, M.; Crhanova, M.; Karasova, D.; Cejkova, D.; Sebkova, A.; Matiasovicova, J.; Faldynova, M.; Pokorna, A.; Cizek, A.; et al. Contact with adult hen affects development of caecal microbiota in newly hatched chicks. *PLoS ONE* **2019**, *14*, e0212446. [CrossRef]
76. Smith, A.M.; Sharma, D.; Lappin-Scott, H.; Burton, S.; Huber, D.H. Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2321–2334. [CrossRef]
77. Zhou, Z.; Yao, H. Effects of Composting Different Types of Organic Fertilizer on the Microbial Community Structure and Antibiotic Resistance Genes. *Microorganisms* **2020**, *8*, 268. [CrossRef]
78. Zhu, P.; Wu, Y.; Ru, Y.; Hou, Y.; San, K.W.; Yu, X.; Guo, W. Industrial-scale aerobic composting of livestock manures with the addition of biochar: Variation of bacterial community and antibiotic resistance genes caused by various composting stages. *Environ. Pollut.* **2022**, *314*, 120270. [CrossRef]
79. Riaz, L.; Wang, Q.; Yang, Q.; Li, X.; Yuan, W. Potential of industrial composting and anaerobic digestion for the removal of antibiotics, antibiotic resistance genes and heavy metals from chicken manure. *Sci. Total Environ.* **2020**, *718*, 137414. [CrossRef]
80. Kubasova, T.; Faldynova, M.; Crhanova, M.; Karasova, D.; Zeman, M.; Babak, V.; Rychlik, I. Succession, Replacement, and Modification of Chicken Litter Microbiota. *Appl. Environ. Microbiol.* **2022**, *88*, e0180922. [CrossRef] [PubMed]
81. Lévesque, C.; Piché, L.; Larose, C.; Roy, P.H. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* **1995**, *39*, 185–191. [CrossRef] [PubMed]
82. Wang, C.; Lin, M.; Yang, Q.; Fu, C.; Guo, Z. The Principle of Steam Explosion Technology and Its Application in Food Processing By-Products. *Foods* **2023**, *12*, 3307. [CrossRef] [PubMed]
83. Simjee, S.; McDermott, P.F.; White, D.G.; Hofacre, C.; Berghaus, R.D.; Carter, P.J.; Stewart, L.; Liu, T.; Maier, M.; Maurer, J.J. Antimicrobial susceptibility and distribution of antimicrobial-resistance genes among Enterococcus and coagulase-negative Staphylococcus isolates recovered from poultry litter. *Avian Dis.* **2007**, *51*, 884–892. [CrossRef]
84. Doyle, M.P.; Busta, F.; Cords, B.R.; Davidson, P.M.; Hawke, J.; Hurd, H.S.; Isaacson, R.E.; Matthews, K.; Maurer, J.; Meng, J. Antimicrobial resistance: Implications for the food system: An expert report, funded by the IFT Foundation. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 71–137.
85. Lee, M.D.; Pedroso, A.A.; Lumpkins, B.; Cho, Y.; Maurer, J.J. Pioneer colonizers: Bacteria that alter the chicken intestinal morphology and development of the microbiota. *Front. Physiol.* **2023**, *14*, 1139321. [CrossRef]
86. Lu, J.; Idris, U.; Harmon, B.; Hofacre, C.; Maurer, J.J.; Lee, M.D. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Appl. Environ. Microbiol.* **2003**, *69*, 6816–6824. [CrossRef]