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Poultry litters with diverse physicochemical and microbial characteristics are associated with significant variation in the degradation of veterinary antimicrobials

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ABSTRACT

The environmental impact of veterinary antimicrobials is assessed through an environmental risk assessment, which includes simulating the degradation of these compounds in manure to refine estimates of environmental exposure. However, manure degradation guidance only applies to liquid manures and current regulations require one source of manure per animal type to be used for these studies. Poultry litter, characterised by a significantly lower water content, is relatively understudied, and the impact of variability in the physicochemical and microbial properties of poultry litter on antimicrobial degradation is poorly understood. Current guidance on the number of litter samples required may not accurately represent the inherent variability in antimicrobial degradation and ultimately the environmental risk posed by these compounds. A series of experiments has been undertaken exploring the impact of poultry litter from various farms with different production strategies on the degradation of five antimicrobials: monensin, salinomycin, sulfadiazine, tiamulin and trimethoprim. Litters from different farms varied significantly in physicochemical properties ($p < 0.05$) and microbial diversity ($p < 0.05$). Measured degradation rates varied significantly ($p < 0.05$) across all farms for all tested antimicrobials, notably for salinomycin, with DT_{50} values (time taken for the compound concentration to reduce by half) ranging from 0.6 to over 10,000 days. These results indicate the need for further research to understand the influence of specific chemical and biological properties of manure on degradation processes. These findings suggest that multiple litters with varying characteristics are needed for manure degradation studies to deliver a robust and realistic environmental risk assessment.

1. Introduction

Antimicrobials, including veterinary medicinal products (VMPs) and feed additives (FAs), are widely used in the poultry industry for the prevention and treatment of disease. Veterinary antibiotics and coccidiostats are some of the most essential veterinary products due to intensive husbandry practices leading to high stocking densities, which increases the risk of disease transmission between animals. Following use, antimicrobials can be excreted in animal manures, and they have been detected at a range of concentrations in different manures from 0.0002 to 91 mg kg^{-1} dry matter content [1], [2].

By 2030, the global consumption of antimicrobials in food-producing animals is projected to reach 200,325 tons [3] despite increasing concerns about antimicrobial resistance (AMR) and the implementation of regulations in many countries to restrict their usage. Up to 90% of an administered antimicrobial is excreted, with these residues dispersing into the environment via the application of manure to land as fertiliser [4]. This contamination poses risks to human health through food and water consumption. Furthermore, negative impacts have been observed at various trophic levels. The uptake of antimicrobial residues into plants can disrupt important physiological processes such as photosynthesis, which has the potential to lead to reduced crop yields and subsequent economic effects [5]. In addition, the presence of antimicrobial residues in the environment results in the exertion of selective pressure

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on microbial communities, promoting the development and spread of AMR [6].

A VMP or FA must undergo an Environmental Risk Assessment (ERA) to be brought to market. An ERA for a VMP in the European Union (EU) comprises two phases [7]. The first phase is used to assess the potential environmental exposure based on the intended use of the VMP and to assess which VMPs may need a more thorough risk assessment, carried out using the Phase II guidance. The Phase II assessment is divided into two tiers, A and B. Tier A requires a mandatory set of empirical data and assesses the risk of a VMP based on exposure and effects in the relevant environmental compartments. If the risk is deemed unacceptable after the Tier A assessment, the process progresses to Tier B for risk refinement. Environmental fate in soil and terrestrial effects studies are carried out during Tier A assessment for intensively reared terrestrial livestock. The assessment requires the calculation of the predicted environmental concentration in soil (PEC_{soil}), which adopts a total residue approach, assuming that the total amount of product administered is excreted. If required, the expected concentrations that will reach the soil environment can be refined using information on the extent of degradation during manure storage on farms to generate a $PEC_{soil\ refined}$ value. The extent of manure degradation can thus be an important factor influencing whether a VMP passes or fails the ERA. Currently, only one manure type per animal category is required to conduct a manure degradation refinement study within the ERA [8]. The ERA process for FAs within the EU follows a similar process using a total residue approach, subsequently allowing PEC_{soil} refinement with manure degradation information [9].

Within the poultry industry, a wide range of farming practices may lead to poultry litter being highly variable in composition due to different bedding types, different feeding regimes and manure management practices [10]. The most common bedding types include wood shavings and straw. This results in litter with varying physicochemical properties such as moisture content, pH, organic carbon and metal contents. Limited research has been carried out to understand the variability of poultry litter and whether this variability may influence the degradation rate of VMPs and FAs. Previous research has shown that variation in pig slurry pH can significantly impact the degradation rates of VMPs [11]. However, poultry litter, a key matrix with its own complex variability, remains critically understudied. Without such data, current risk evaluations may underestimate or overestimate the persistence of these substances in the environment.

The selected compounds, monensin, salinomycin, sulfadiazine, tiamulin and trimethoprim, represent a broad spectrum of antibiotic classes commonly used in poultry production. Notably, the inclusion of ionophore antibiotics (monensin and salinomycin), which are classified as FAs rather than therapeutic antibiotics, allows for an exploration of substances that are regulated differently yet remain integral to poultry health management, particularly for the prevention of coccidiosis. This parasitic disease poses significant risks to poultry welfare and farm profitability, necessitating the prophylactic use of ionophores, the only antibiotics permitted for such use. Conversely, therapeutic antibiotics like sulfadiazine (often used in combination with trimethoprim) and tiamulin are employed to treat bacterial infections, including those caused by *Escherichia coli*, *Salmonella* spp. and *Staphylococcus* spp., as well as mycoplasma-related diseases. Potentiated sulfonamides (e.g. sulfadiazine-trimethoprim combinations) accounted for 7% of antibiotic use in broilers in 2023 [12], underscoring their continued relevance. This research, therefore, aims to fill a crucial gap by linking litter variability, arising from diverse production and management practices, to VMP and FA degradation, providing essential data to inform risk assessments and support more sustainable waste management practices in poultry production.

2. Materials and methods

2.1. Chemical compounds and stock preparation

Analytical grade antibiotics, including salinomycin sodium, tiamulin hydrogen fumarate, and trimethoprim, were purchased from VWR (USA). Monensin sodium was purchased from Fisher Scientific (USA). Sulfadiazine sodium was purchased from Sigma-Aldrich (USA). A summary of physical properties of the selected compounds is shown in Table S1, which can be found in Appendix A.

The dose was calculated as 0.2 mg kg^{-1} for all compounds, as this is lower than 10% of the highest calculated predicted environmental concentration in manure (PEC_{manure}) and this dose was tested in a pilot degradation study (data not shown) to ensure that the test antibiotics could be detected at the chosen concentration. These values are within the range of measured environmental concentrations reported in the literature [1], [2]. The predicted environmental concentrations were calculated using the equations given in the EMA and EFSA guidance [13].

2.2. Poultry litter sampling, conditioning and characteristics

A total of 45 kg of poultry litter was collected from three farms (15 kg collected per farm) in the United Kingdom (UK). The litter was collected from one shed per farm. The litter was not reused from any previous flocks on any of the farms and was not treated with any amendment prior to collection. The farms were labelled A, B and C and the birds at the time of litter collection were 49 weeks, 23 days and 12 days old, respectively. Farm A housed broiler breeders, and Farms B and C housed broilers. All birds were the same strain and breed, Ross 308 birds. Different production types and bird ages were selected to ensure that the variability of litter types collected for regulatory manure degradation studies was captured for comparison. None of the study compounds had been administered before collection, and the litter was stored under aerobic conditions at room temperature following collection for no longer than four weeks before use, in line with the regulatory guidance that states that litter must be stored prior to use in the study. The litter was homogenised using a Kenwood Food Processor for 5 min at room temperature. All collected litter was homogenised before being weighed into the test vessels. The moisture content was determined gravimetrically and adjusted as necessary using Type 1 water with a resistivity of 18.2 MΩ-cm (ELGA LabWater, UK) to maintain a dry matter content of 40 ± 5% dry weight (d/w).

The total carbon, organic carbon and nitrogen contents of the litters were determined using a vario MICRO cube (Elementar, UK). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine the acid extractable metals. The microbial activity of the litters was measured using an Ensure® Touch Luminometer (Hygeina®, USA) and pH was measured using a hand-held meter (Oxitop®, UK). Three biological replicates were used to determine pH and microbial activity. Each biological replicate was also measured three times to give three technical replicates.

2.3. Degradation kinetics

2.3.1. Experimental design

A mixed stock solution was prepared by initially dissolving the test compounds entirely in methanol to obtain a concentration of 1 mg mL^{-1}. The application solution was prepared from this stock solution by diluting with Type 1 water to minimise the effect of the solvent on the microbial communities present in the litter.

Eleven time points were used to determine degradation kinetics: Days 0, 2, 4, 6, 13, 24, 35, 49, 63, 90 and 120. These timepoints were selected due to a mixture of VMPs and FAs being investigated with concentration data being collected before and after the DT_{50} to ensure accurate kinetic modelling, up to the maximum time limit of 120 days,

as per the regulatory guidance. Five replicate treatments, two quality controls and one blank sample were prepared. The quality control samples were used to verify the extraction efficiency throughout the study. Blank litter samples consisted of litter that was dosed with 1 mL of Type 1 water. 50 g (dry weight) of litter was weighed into each test vessel and dosed with 1 mL of the application solution to give a concentration of 0.2 mg kg^{-1}. Each sample was mixed with a spatula to ensure homogeneity. During incubation, the vessels were maintained at 20 ± 2 °C in the dark at a dry matter content of 40 ± 5% (d/w) (monitored gravimetrically), as per the regulatory guidance for manure degradation studies. The temperature of the room where the samples were stored was monitored using a Tinytag datalogger (Gemini Dataloggers, UK).

2.3.2. Chemical extraction and analysis

After a pre-incubation period of 21 days, as suggested in the regulatory guidance, 100 mL of acetonitrile was added to each sample (50 g/100 mL) and shaken for 5 s by hand before being placed on a vortex mixer for 1 min. Samples were then placed in an ultrasonic bath for 10 min at 20 °C and shaken on an orbital shaker at 300 rpm for 45 min before being centrifuged at 3724 RCF for 20 min at 4 °C. The supernatant was removed and transferred into a 2 mL Eppendorf™ tube and ultracentrifuged at room temperature for 5 min at 20,000 RCF. Samples were then transferred into amber 2 mL High Performance Liquid Chromatography (HPLC) vials and frozen at −20 °C before analysis. The extraction and liquid chromatography tandem mass spectrometry (LC-MS/MS) method was validated in accordance with the SANTE/2020/12830 rev.2 [14] and VICH GL2 guidelines [15] and included evaluation of the linearity, limits of detection (LOD), limits of quantification (LOQ), recovery, repeatability and selectivity of the method (Appendix E in supplementary information). The LOD ranged from 0.0183 ng mL^{-1} for tiamulin to 3.86 ng mL^{-1} for sulfadiazine, and the LOQ ranged from 0.0554 ng mL^{-1} for tiamulin to 12.9 ng mL^{-1} for sulfadiazine. Matrix-matched calibration standards were prepared following an extraction of blank poultry litter samples to compensate for matrix effects. For quantification, an eight-point linear calibration curve was generated for a calibration range between 1 ng mL^{-1} and 150 ng mL^{-1}.

Detection and quantification of the test compounds were achieved using both positive and negative reverse-phase methodologies on a Triple Quad 5500+ LC-MS/MS (SCIEX, USA). An ACQUITY UPLC HSS T3, 100 Å, 1.8 μm, 2.1 by 100 mm column (Waters, USA), maintained at 40 °C, was used for both methods. The injection volume was 1 μL. Data processing and quantitation were performed using Analyst 1.6 software (SCIEX, USA). The positive mode was used to detect and quantify sulfadiazine, tiamulin, and trimethoprim, and a gradient elution was used at a flow rate of 0.3 mL min^{-1}. The mobile phase used for this positive mode was (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The negative mode was used for the detection and quantification of monensin and salinomycin. The mobile phase used for the negative mode comprised (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile and methanol (50:50 v/v). For further information on the gradients used, please refer to the supplementary material (Appendix B).

The kinetic profile [13] of each test compound was derived using Computer Assisted Kinetic Evaluation (CAKE v3.7) modelling software. The best-fit kinetic models were independently selected for each compound and each litter. Statistical analysis was carried out using R and RStudio software. The three litter types were statistically compared using a Kruskal-Wallis test with a Bonferroni-Holm adjustment. Five replicates were used to conduct statistical analysis, which was reported at the 95% confidence interval ($p < 0.05$).

2.4. Metabarcoding analysis

Litter collected from the farms was handled and collected into vessels and homogenised using a Kenwood Food Processor for 5 min at room temperature. One sample was collected for metabarcoding analysis at three timepoints across the experiment (Days 0, 61 and 120) for each farm (A, B and C) and stored at −20 ± 2 °C prior to deoxyribonucleic acid (DNA) extraction.

DNA extractions were performed on defrosted samples (three biological replicates per timepoint and farm) with the QIAmp DNA Power Soil Kit (Qiagen, Germany) according to the protocol provided by the manufacturer. Briefly, buffer was added to a sterile universal containing 250 mg of previously homogenised poultry litter and the mixture underwent bead beating using a vortex at maximum speed for 10 min. This was followed by centrifugation and purification using silica membrane spin columns. Extracted DNA was stored at −20 °C until sequencing analysis, and nine poultry litter samples from Farms A, B and C were used for 16S ribosomal ribonucleic acid (rRNA) and Internal transcribed spacer (ITS) gene-based microbial community analysis. Polymerase chain reaction (PCR) amplification was performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA) to amplify the V4 region of the 16s rRNA gene for bacteria and the ITS1 region for fungi. For further information on the primers used, please refer to the supplementary material (Appendix C). The following cycling conditions were used for 16S samples: initial denaturation at 98 °C for 2 min, 98 °C for 20 s, followed by 22 cycles of a reduction of 0.5 °C per cycle from 65 °C to 54 °C for 45 s, 72 °C for 60 s, 98 °C for 20 s, 8 cycles of 54 °C for 45 s, 72 °C for 60 s, with a final extension of 72 °C for 10 min. ITS cycling conditions were as follows: 98 °C for 2 min, 98 °C for 20 s, followed by 25 cycles of 54 °C for 30 s, 72 °C for 90 s, with a final extension at 72 °C for 5 min. The quality of the PCR samples was confirmed using gel electrophoresis. Amplicons were purified using AMPure XP magnetic beads (Beckman Coulter, USA), and indexing was carried out in a second PCR step. Final libraries were quantified using a Qubit 4 Fluorometer (Invitrogen, USA) and assessed for fragment size and quality using the Agilent TapeStation System (Agilent, USA). The libraries were normalised, pooled equimolarly, and sequenced using an Illumina MiSeq platform (Illumina, USA) using a V3 MiSeq reagent kit (Illumina, USA) to obtain 2 x 300 bp paired-end reads. A positive control was used to confirm the efficacy of the PCR and sequencing methods. An artificial positive control was used that was designed with binding sites for all major PCR primers used at the facility. Negative controls of Molecular Grade Biology Water were used to assess reagent contamination at PCR and indexing stages to confirm there was no contamination. Extraction blanks of extraction buffers taken throughout the process were used to confirm that the extraction process did not introduce sample contamination. One limitation of the methods used in this study is that the negative controls used do not account for the potential introduction of microbial taxa at upstream stages, such as during litter collection, handling, storage and homogenisation. Control data are presented in Appendix F in the Supplementary information. The negative controls and extraction blanks were filtered out as part of quality control and trimming, due to the very low read count. An additional limitation of the methods used is that a mock microbial community control was not used. The authors acknowledge that DNA extraction methods can have significant impacts on metabarcoding results and are one of several sources of bias inherent to the method.

16S rRNA and ITS results were analysed separately. Raw reads were processed using the QIIME2 software package (version 2024.5) and are available on the SRA database [16]. Quality control and denoising were performed using the Divisive Denoising Algorithm 2 (DADA2), which utilises a model-based approach to infer true biological sequences from reads and remove chimeras and low-quality sequences. Dereplication of the reads and inference of the Amplicon sequence variants (ASVs) was subsequently carried out. A pre-trained naïve Bayes classifier for the V3-V4 region of the 99% SILVA v.128 database was used to classify representative sequences [17], [18], [19]. The ITS classifier used was composed of NCBI RefSeq Target Loci ITS database sequences with additional Oomycete sequences from the Unite database [20], [21], [22].

A multiple sequence alignment of the sequences was carried out on the ASVs using the Multiple Alignment Fast Fourier Transform (MAFFT) program. The aligned ASVs were then placed into a phylogenetic tree using FastTree. Samples were rarefied to the minimum depth of sequences observed in any given sample. Metrics of alpha diversity (observed ASVs, Faith's Phylogenetic Diversity, Shannon's Diversity Index and Pielou's evenness) and beta diversity (Bray-Curtis distance, Jaccard distance, unweighted UniFrac distance and weighted UniFrac distance) were estimated.

An Analysis of Composition of Microbiomes (ANCOM) test was carried out to determine if there were any significant differences in the relative abundance of any taxa between farms. QIIME2 was used to carry out statistical analysis for alpha and beta diversity metrics. A Kruskal-Wallis test was used to determine if there was a significant difference in alpha diversity between all the groups and for pairwise comparisons between farms. Two replicates (n = 2) were used to conduct statistical analysis, and statistical significance was reported at the 95% confidence interval ($p < 0.05$).

2.5. Antimicrobial susceptibility testing

One treated replicate and one blank sample were prepared for each farm (A, B and C) at three timepoints (Days 0, 61 and 120) for antimicrobial susceptibility testing. At each time point, one 10 g aliquot was taken from each blank sample, and two 10 g aliquots were taken from each treated replicate. Each aliquot was added to a sterile universal tube, and 90 mL of Maximum Recovery Diluent (Thermo Fisher Scientific, USA) was added and vortexed to mix. Appropriate dilutions were prepared, and 0.1 mL from each dilution was inoculated and spread onto a Slanetz and Bartley agar plate (Thermo Fisher Scientific, USA) and incubated aerobically at 37 ± 1.7 °C for 48 h 0.1 mL from each dilution was inoculated and spread onto a Modified Charcoal Cefoperozone Deoxycholate agar plate (mCCDA) (Thermo Fisher Scientific, USA) and incubated at 41.5 ± 0.9 °C in a glass jar containing a microaerophilic sachet for 48 h 0.1 mL from each dilution was inoculated and spread on a Tryptone Bile X-glucuronide (TBX) agar plate (Thermo Fisher Scientific, USA) and incubated at 37 ± 1.7 °C for 4 h and then transferred to an incubator set at 43.5 ± 0.9 °C for a total incubation time of 44 h. The incubation times and temperatures were selected in line with the standard operating procedures for each of the selective agar plate types. After incubation, *Enterococcus* spp. (pink on Slanetz and Bartley agar), *Campylobacter* spp. (grey on mCCDA agar) and *E. coli* (blue on TBX agar) colonies were identified and counted. A maximum of five presumptive colonies were randomly selected to ensure a representative sample and transferred into 1 mL sterile Eppendorf™ tubes containing 10 μL of horse serum and stored at −80 °C until susceptibility testing. When fewer than five colonies were available for collection due to limited growth, the maximum number of colonies available were selected and transferred to ensure the sample was representative.

The antimicrobial susceptibility testing of isolates was conducted using an agar well diffusion method to investigate differences in resistance profile between the litter samples from different farms. Due to no breakpoints being available for the test compounds for *Enterococcus* spp., isolates could not be determined as clinically resistant or susceptible, so the zone diameter was reported to indicate differences in the resistance profile between isolates [23], [24].

3. Results

3.1. Poultry litter characteristics

Poultry litter showed high variability in its physicochemical characteristics (Table 1), with significant differences between farms depending on the specific measurement. Farm A and Farm B varied significantly ($p < 0.05$) in pH, total nitrogen, total carbon, total organic carbon, arsenic concentration and zinc concentration. Farm A and Farm C varied significantly in total nitrogen content, total carbon, total organic carbon, zinc, copper and arsenic concentrations. In contrast, Farms B and C differed significantly in copper and zinc concentration and total nitrogen content.

Table 1
Summary of the physicochemical characteristics of poultry litter samples from different farms.

Physicochemical Characteristic	Farm A	Farm B	Farm C	p value[a]
Production type	Broiler Breeder	Broiler	Broiler	n/a
Bedding material	Wood shavings	Wood shavings	Wood shavings and straw mix	n/a
pH	8.86	9.16	9.25	7.22×10^{-10} ***
Microbial activity (lux)	15031	10485	10699	0.372
Total Nitrogen (%)	2.75	3.31	2.24	3.05×10^{-7} ***
Total Carbon (%)	35	41.5	42.8	0.000303***
Total Organic Carbon (%)	31.6	38.6	38.4	1.4 x 10-5***
Copper Concentration (mg kg^{-1})	87.2	92.4	51.8	0.0000140***
Zinc Concentration (mg kg^{-1})	412	437	254	0.000000420 ***
Arsenic Concentration (mg kg^{-1})	0.319	0.2	0.178	0.000174***

Statistical differences between farms are given as *p < 0.05, **p < 0.01, and ***p < 0.001.

3.2. Degradation kinetics

Significant differences in the concentration of each compound at each time point and farm were observed, apart from tiamulin, which only differed significantly from day 6 onwards. This is reflected in the varied degradation rates calculated in this study, particularly for salinomycin and trimethoprim (Table 2). In the context of this study, degradation is defined as a reduction of extractable parent residue over time and reported as the DT_{50}, which is the time taken for the concentration to reduce by half. Although the routes of dissipation were not determined, they were assumed to be sorption to the litter matrix, degradation to a metabolite or mineralisation.

Salinomycin degradation varied substantially with concentrations being significantly different between all farms at six time points; Day 2 ($p = 0.0246$), Day 4 ($p = 0.0374$), Day 6 ($p = 0.0193$), Day 24 ($p = 0.0193$), Day 35 ($p = 0.0306$), Day 120 ($p = 0.0193$) (Fig. 1a), and much slower in Farm A samples than Farm B and Farm C samples. Substantial variation was also observed for trimethoprim with significant

Table 2
DT_{50} values per farm for each veterinary antibiotic and feed additive per farm.

Compound	DT_{50} (days)			Pairwise comparisons with statistically significant differences
	Farm A	Farm B	Farm C	
Monensin	20.8	18.4	7.43	A-C
Salinomycin	>10000	9.84	0.649	A-C
Sulfadiazine	0.676	2.01	1.45	A-B
Tiamulin	53.1	27.1	18.7	B-C
Trimethoprim	104	101	37.2	B-C

Statistical comparison was conducted using a Kruskal-Wallis test for each compound and time point. Dunn tests were carried out for pairwise comparisons and included if over half the time points showed a statistically significant difference.

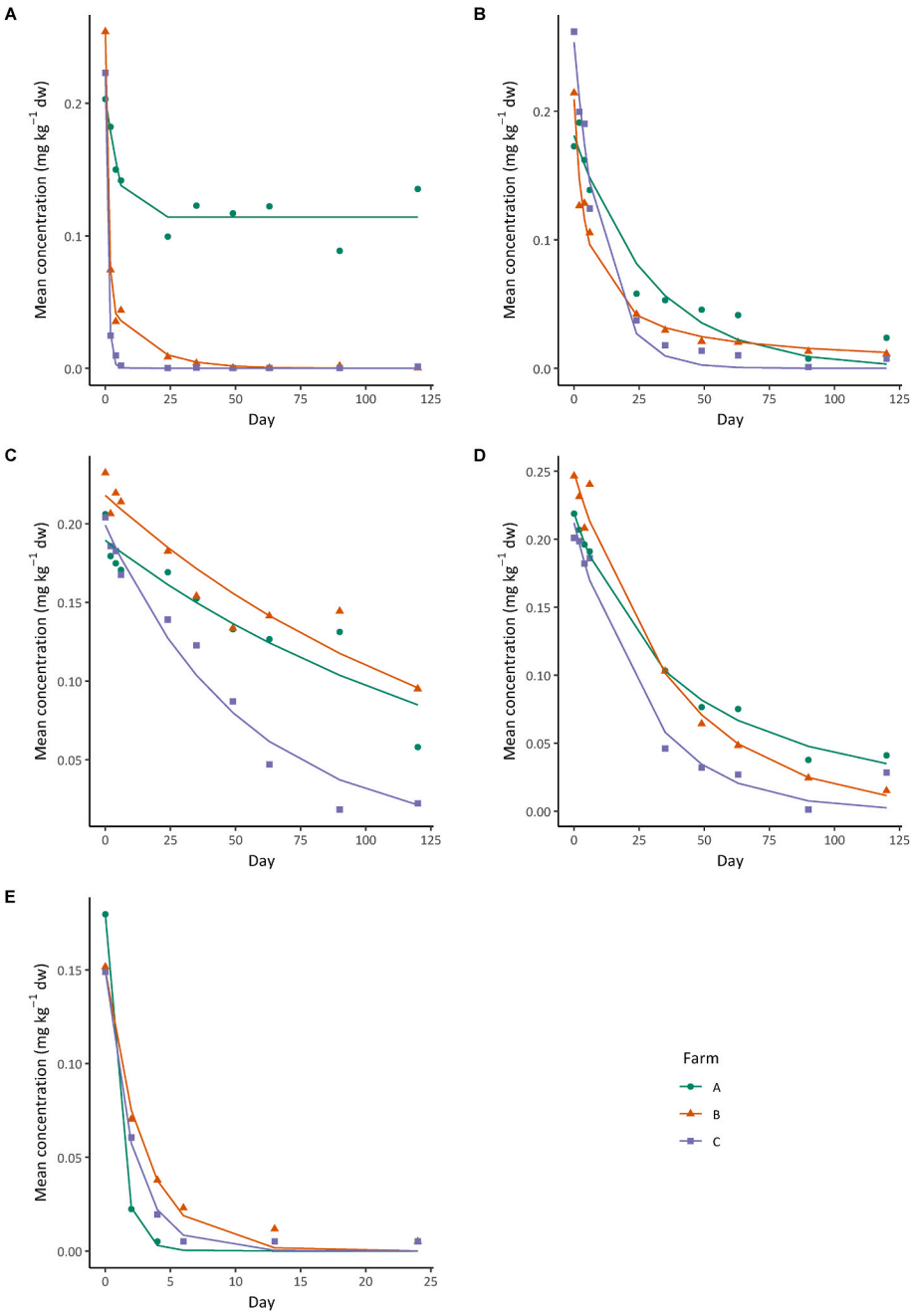

Fig. 1. Temporal trends in the mean concentration (n = 5) of five veterinary medicines and feed additives, salinomycin (A), monensin (B), trimethoprim (C), tiamulin (D) and sulfadiazine (E), in poultry litter samples collected from Farms A (green circles), B (orange triangles), and C (purple squares). Concentrations (mg kg^{-1} dry weight) are plotted over a 120-day study period. The data illustrate compound specific degradation profiles and inter-farm variability in persistence.

differences in concentration at five time points; Day 2 ($p = 0.0336$), Day 4 ($p = 0.0492$), Day 24 ($p = 0.0270$), Day 63 ($p = 0.0336$), Day 120 ($p = 0.0212$). However, in contrast to salinomycin, trimethoprim degradation was much slower in Farm A and Farm B litter, compared to Farm C (Fig. 1c). In contrast, sulfadiazine, tiamulin and monensin showed less variation in degradation rates, with each farm having DT_{50} values within 14 days of each other.

The DT_{50} values determined from manure degradation studies are essential for calculating $PEC_{soil\ refined}$ during the ERA of VMPs and FAs. To understand the effect of variable degradation rates on $PEC_{soil\ refined}$, the DT_{50} values determined in this study were incorporated into the calculation. Results revealed substantial variations in PEC_{soil} refinement when considering litter from different farms (Fig. 2). $PEC_{soil\ refined}$ values ranged from 1.15×10^{-6} μg kg^{-1} for sulfadiazine (Farm A) to 1060 μg kg^{-1} for salinomycin (Farm A). Using the DT_{50} value for salinomycin data from Farm C samples led to a 99.9% decrease in $PEC_{soil\ refined}$ compared to Farm A. In comparison, when comparing $PEC_{soil\ refined}$ values from Farm A against Farm C, there was only a 20% decrease.

3.3. Metabarcoding analysis

Significant differences in the alpha diversity of litter samples from different farms were identified. The Faith's Phylogenetic Diversity (Faith's PD) of the bacterial communities present in the litter was significantly different between farms ($p = 0.0154$) but only between Farm A and Farms B ($p = 0.0176$) and C ($p = 0.0283$). The average Faith's PD index for Farm A was higher than the other two farms, which implies that the bacterial community present in the litter from Farm A

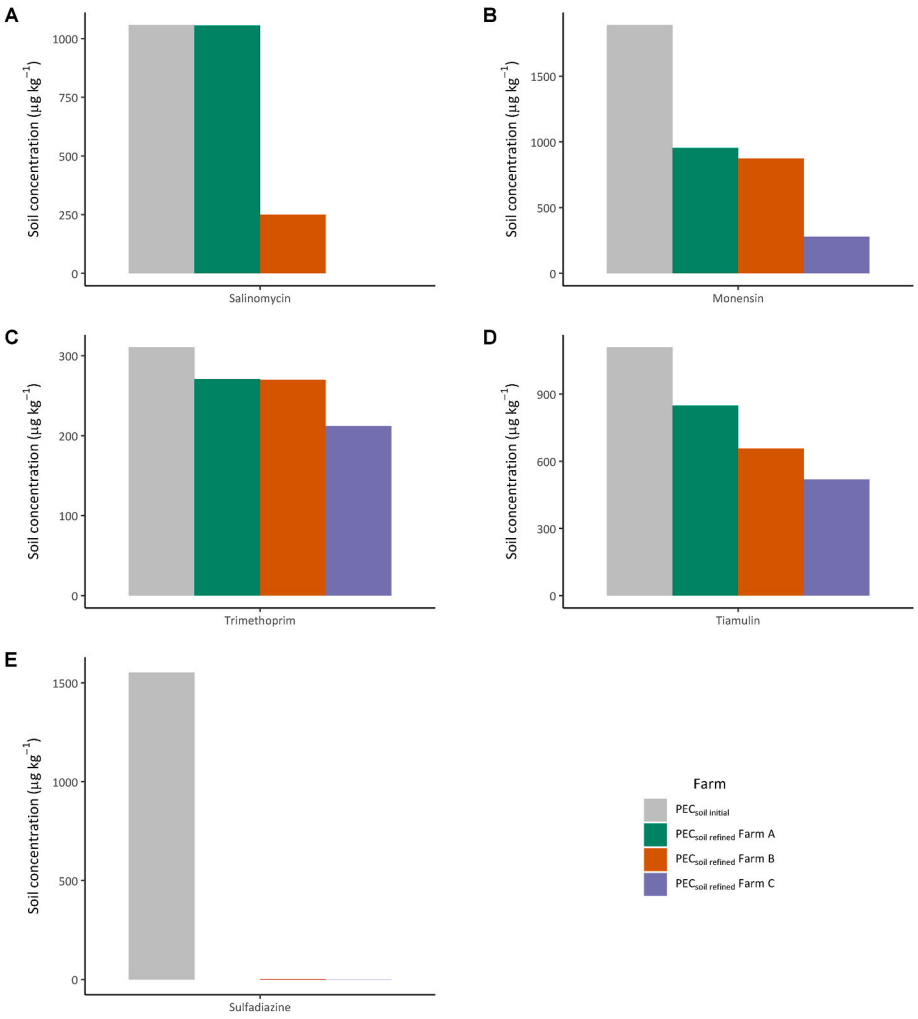

Fig. 2. Comparison of the $PEC_{soil\ initial}$ and $PEC_{soil\ refined}$ values of five veterinary medicines and feed additives (salinomycin (A), monensin (B), trimethoprim (C), tiamulin (D) and sulfadiazine(E)) calculated using DT_{50} values for poultry litter samples collected from Farms A (green), B (orange) and C (purple). Bars represent the predicted soil concentration in µg kg^{-1} (dry weight), highlighting discrepancies between modelled and observed values across sites and compounds. Plots for salinomycin (A) and sulfadiazine (E) appear to have fewer bars due to the calculated $PEC_{soil\ refined}$ values being much lower than the $PEC_{soil\ initial}$ and close to zero which makes it appear as if there is no bar.

was more diverse. In contrast, the Faith's PD of the fungal communities did not differ significantly by farm ($p = 0.205$). However, the Shannon Diversity results for fungal communities differed significantly between farms ($p = 0.0380$), suggesting variation in both species richness and evenness. The Shannon Diversity only differed significantly between Farm A and Farm C ($p = 0.0285$). The average Shannon Diversity was lower for Farm C, suggesting that the fungal community is less diverse than that in Farm A or Farm B litter. When comparing the differences in alpha diversity metrics of both bacterial and fungal communities pre- and post-dosing, there were no significant differences between the groups for all alpha diversity metrics calculated (Faith's PD, Shannon Diversity, Pielou's evenness and Observed ASVs; $p > 0.05$).

Beta diversity analysis showed statistically significant differences between the litter samples between different farms (Bray-Curtis distance; 16S, $p = 0.004$, ITS, $p = 0.001$). Pairwise comparisons revealed statistically significant differences in the beta diversity of the bacterial communities between the litter samples from Farms A and B and A and C (Bray-Curtis distance; Farm A and B $p = 0.037$, Farm A and C $p = 0.011$). Principal Coordinate Analysis (PCoA) (Figs. 3 and 4) was conducted to visually assess the relationships between beta diversity parameters. For both 16S and ITS results, most of the variance in Bray-Curtis distance or the dissimilarity in bacterial or fungal species composition between sites was explained by time, which aligns with expectations as communities

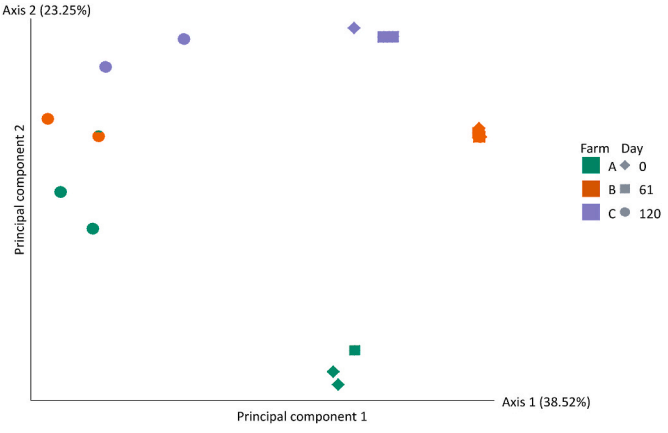

Fig. 3. Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarity for bacterial 16S rRNA gene profiles from poultry litter samples collected at Farms A, B and C over time. Axis 1 and Axis 2 explain 38.52% and 23.25% of the variation, respectively. Colours and symbols represent farms and time points: green (Farm A), orange (Farm B), purple (Farm C), diamonds (day 0), squares (day 61) and circles (day 120), indicating microbial community shifts with time and between sites.

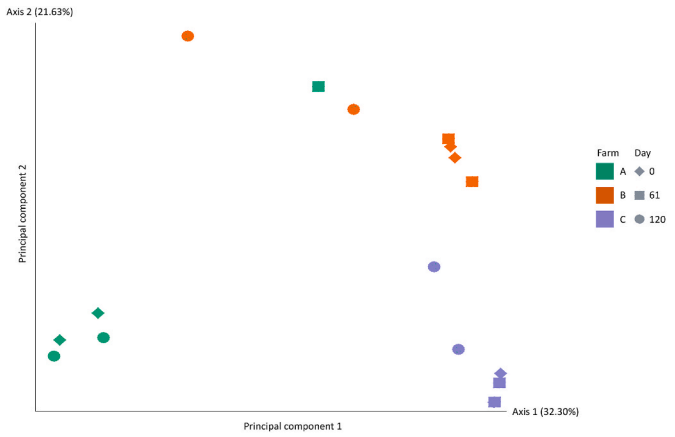

Fig. 4. Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarity for fungal ITS gene profiles from poultry litter samples collected at Farms A, B and C over time. Principal component 1 (Axis 1) and 2 (Axis 2) explain 32.30% and 21.63% of the total variance, respectively. Data points are colour-coded and shaped according to farm and sampling day: green (Farm A), orange (Farm B), purple (Farm C), diamonds (day 0), squares (day 61) and circles (day 120), demonstrating clustering patterns and temporal shifts in fungal profiles.

were predicted to change over time. However, 23.25% (16S) and 21.63% (ITS) of the variance was attributed to differences between farms, as further supported by permutational multivariate analysis of variance (PERMANOVA) results for the Bray-Curtis distance ($p < 0.05$).

The most abundant genera found in the poultry litter samples were *Brachybacterium*, *Corynebacterium* and *Staphylococcus*, which contain pathogenic and non-pathogenic species and are commonly found in poultry manure (see Fig. S1 in Appendix D of the Supplementary Information). *Enterococcus*, *Pseudomonas* and *Escherichia/Shigella* were examples of genera detected in the study samples that are known to contain species that are potential pathogens; however, due to the resolving power of 16S metabarcoding, further details on the presence of pathogens could not be determined. Farm B had the highest relative abundance of staphylococci compared to Farms A and C. However, *Gammaproteobacteria* were much more abundant in Farm C than in the other two farms. *Actinobacteria* were prevalent in all farms, but the levels varied between farms. *Lactobacillales*-related bacteria were high in Farms A and B but low in Farm C, whilst *Saccharimonadaceae* were rare in Farms A and B but abundant in Farm C. Some of the classes and genera identified in these samples are associated with the degradation and resistance of the test compounds investigated in this study, including *Actinobacteria*, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Comamonas* and *Streptomyces*.

3.4. Antimicrobial susceptibility testing

No *E. coli* or *Campylobacter* spp. were isolated from the poultry litter samples; however, enterococci were isolated from all samples. As clinical breakpoints for the test compounds in *Enterococcus* were unavailable, the isolates could not be classified as clinically resistant or susceptible. Instead, the zone of clearance was used to make comparisons between sites. There were significant differences between the zones of clearance for tiamulin ($p = 0.0213$) (Fig. 5d), although all other test compounds showed no significant differences in zone of clearance between farms (Fig. 5).

When comparing the zones of clearance for each compound between

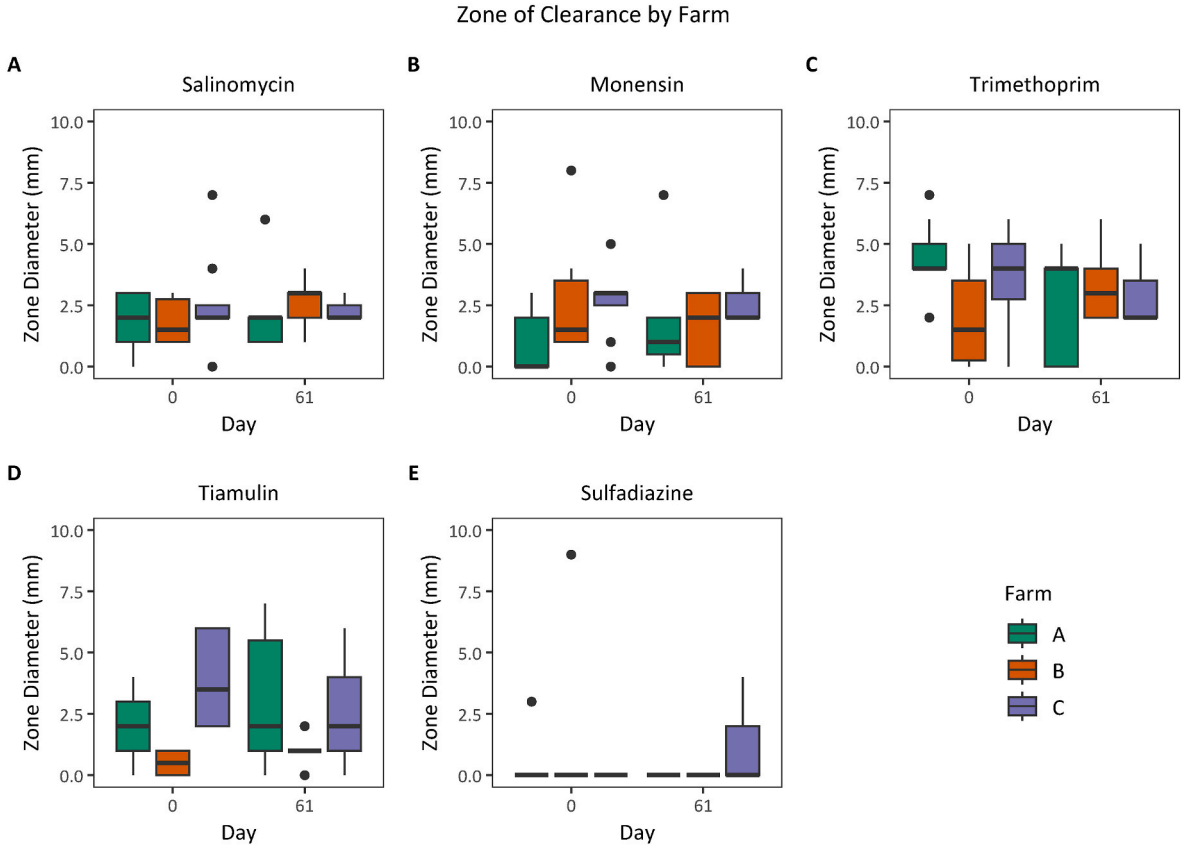

Fig. 5. Box plots showing zone of clearance diameters (mm) produced by five different veterinary medicines and feed additives (salinomycin, monensin, trimethoprim, tiamulin and sulfadiazine) in enterococci isolates from poultry litter samples from Farms A (green), B (orange) and C (purple) at Day 0 and Day 61 post-application. Each box represents interquartile ranges, with whiskers indicating variability and median lines showing central tendency. The dataset compares temporal changes and inter-farm differences in antibacterial activity, with larger zones reflecting greater inhibitory effect.

day 0 and day 61 (Fig. 5e), there was a significant difference between the wells containing sulfadiazine for Farm C isolates ($p = 0.0455$). However, there were no significant differences between the zones of clearance for any other isolates.

4. Discussion

4.1. Poultry litter characteristics

Several factors may drive the significant differences observed in the physicochemical properties of the litter, and these differences may impact the degradation rate of different VMPs and FAs. Fresh litter materials usually have pH levels near neutral, but change as excreta accumulates, which can be influenced by the bedding material used. For example, wood shavings tend to maintain a more neutral pH. In contrast, straw and hay initially become more acidic as they decompose due to organic acids produced during microbial decomposition. They can, however, become more alkaline as the decomposing straw releases alkaline cations, which neutralise H^+ ions and increase the pH [25]. pH influences the chemical stability of antibiotics, and some will degrade faster in alkaline conditions, while others may be more stable at neutral or acidic pH. This may be one of the factors driving the differences in degradation rate of some of the test compounds in this study (Fig. 1). For example, litter from Farms B and C had higher pH values than Farm A, which may explain the more rapid degradation of tiamulin in Farm B and Farm C litter compared to Farm A (Fig. 1d). This may be due to the chemical structure and behaviour of the molecule; the ester linkage present in tiamulin is prone to hydrolysis, especially under alkaline conditions due to the nucleophilic attack by hydroxide ions. In contrast, sulfadiazine is a sulfonamide antibiotic, and this appeared to be more stable in the alkaline conditions of the litter from Farms B and C with more rapid degradation observed in Farm A samples (Fig. 1e), which aligns with the findings from the previously reported literature [11].

4.2. Degradation kinetics

As previously described, manure degradation studies are used as risk refinement tools in the ERA of VMPs and FAs. Several methodologies can be used to conduct a manure degradation study, and manure degradation data for PEC_{soil} refinement can also be obtained using techniques such as the calculation of the fraction active using radiolabelled material, such as 14-C. In contrast to the soil degradation study guidelines, manure degradation studies only require a single manure type to be used to assess the degradation of the product in manure. There are several ways in which the PEC can be refined, one of which is refinement based on degradation in manure. The length of time the manure is stored is calculated as half the total manure storage time to account for variation in the point during the housing period in which the animals are treated. If the animals are treated at the beginning of the storage period there will be more time for the active ingredient to degrade. The $PEC_{soil\ refined}$ is then compared to the Predicted No-Effect Concentration (PNEC) to calculate the risk quotient (RQ). The ERA can be concluded at Phase II Tier A if the RQ values are <1. Soil is the primary exposure route, and the calculations of both PEC_{soil} and $PEC_{soil\ refined}$ impact the quantified risks to other environmental compartments. The DT_{50} value generated in the manure degradation study is also used in the ground and surface-water models. Therefore, any inaccuracies in the DT_{50} values would lead to inaccurate $PEC_{soil\ refined}$ values, which could impact the outcome of the ERA as a whole and lead to products being incorrectly approved or denied market authorisation. The results from this study highlight the potential variability in the DT_{50} and subsequently in the $PEC_{soil\ refined}$ values, as seen in Fig. 2, with some values varying by up to ten orders of magnitude. In the case of salinomycin, the RQ would be 1.03 if litter from Farm A was used to refine the PEC. As the RQ > 1, this would trigger further testing or denial of market authorisation. However, if the litter from Farms B or C were used, the RQ values would be 0.00138 and 2.88×10^{-10}, respectively, allowing for completion of the risk assessment and approval of the product to go to market.

Limited information has been published on the DT_{50} values of the test compounds used in this study, particularly in poultry litter. However, comparison with those produced in the current study suggests that degradation can vary (Table 3). Berendsen et al. (2018) reported a DT_{50} for tiamulin of 280 days, which suggests that tiamulin is much more persistent in some litter than in those tested in this study, which had a longest DT_{50} of 33.1 days. This further highlights the variability of poultry litter and the critical need to understand what drives this variability to improve the accuracy of the ERA. Further research is required to get a more comprehensive understanding of the degradation of VMPs used in the poultry industry and the extent to which these differences vary. Further investigation into the specific parameters that drive these differences should be carried out so that adjustments can be made to the ERA guidance to ensure that this variation is accounted for and products are not incorrectly granted or denied market authorisation.

4.3. Metabarcoding analysis

Differences in microbial community composition of litter will influence the degradation rate of different VMPs and FAs, depending on the taxonomy and relative abundance of the microbes present. Antibiotrophs are different from other resistant bacteria because they can neutralise and catabolise antibiotics for nutrition and cellular homeostasis. Agricultural and healthcare environments are likely to harbour the most elevated populations of antibiotrophs. Several known or suspected antibiotrophs were identified in the samples in this study, including *Pseudomonas*, *Microbacterium*, and *Achromobacter* [29], [30], [31]. If different manures have varied abundances of these antibiotrophic bacteria, this may influence the rate of degradation of the antibiotics that the bacteria can subsist on. The relative abundance of *Pseudomonas* was much greater in Farm C samples compared to Farms A and B, which may influence sulfadiazine degradation. The average relative abundances of *Achromobacter* and *Microbacterium* were similar in Farms B and C; however, no *Achromobacter* was present in Farm A samples. Co-metabolism is when bacteria degrade antibiotics whilst feeding on another carbon source, in contrast to antibiotrophs, which use the antibiotic as the sole carbon source. Examples of genera of bacteria detected in the study samples that have known co-metabolism with the test compounds include *Bacillus*, *Staphylococcus* and *Escherichia* [32]. *Bacillus* had the highest abundance in Farm B samples, closely followed by Farm C and the least abundant in Farm A litter. *Escherichia* and *Staphylococcus* were most abundant in Farm B samples, less abundant in Farm A samples and least abundant in Farm C. The detection of *Escherichia* using 16S rRNA sequencing highlights a potential disagreement between the 16S rRNA sequencing and culture results, as no *E. coli* were isolated from the litter samples. This may be due to several reasons. Firstly, the DNA that was sequenced may have been free DNA from dead cells, as metabarcoding is unable to distinguish between live and dead cells. Alternatively, the species present may not have been *E. coli*, but a related species, for example, *Escherichia albertii*. This species has been isolated from poultry environments globally [33] and wild birds in the UK [34] but lacks the ability to produce β-D-glucuronidase [35] which is responsible for the colour change of *E. coli* on TBX agar. However,

Table 3
Summary of literature DT_{50} values for selected veterinary medicines and feed additives.

Compound	DT_{50} value (days)	Matrix	Reference
Monensin	7.4	Cattle manure	[26]
Salinomycin	5.1 ± 0.3	Pig manure	[27]
Sulfadiazine	4.4	Broiler manure	[28]
Tiamulin	280	Broiler manure	[28]
Trimethoprim	31.5	Sewage sludge	[26]

atypical colonies were not retained so this could not be further investigated. Finally, this may be due to the heterogeneous nature of the poultry litter, where *E. coli* was genuinely present in samples taken forward for DNA extraction, but not for culture. For example, enterococci were isolated from all samples but were not detected in metabarcoding data for all samples, although this may be due to differences in sensitivity of the two methods. These results show that alpha diversity significantly differs between farms, as poultry litter is a reflection of gut microbiota and environmental microbes. Beta diversity highlights the extent of the differences in taxa between the microbial community in Farm A samples compared to Farms B and C, even if both have similar richness (Figs. 3 and 4). Beta diversity is influenced by similar factors to alpha diversity. The greater the difference in beta diversity, the more different these factors are between farms. Significant differences in the Faith's PD between farms suggest that bacterial communities in poultry litter may vary in the diversity of their ecological roles and adaptations. A higher value of Faith's PD indicates a more stable and resilient ecosystem due to more diverse evolutionary backgrounds, which provide the means to act as a buffer to environmental changes. The significant differences in Faith's PD may be attributable to the differences in the ages of the birds between the different farms; however, this requires further investigation. In contrast, the fungal communities in the litter were not significantly different regarding Faith's PD, but according to the Shannon diversity index. This suggests differences in the taxonomic richness and evenness within the fungal communities of the different litter types. The lower average Shannon diversity observed in Farm C may be attributable to the younger age of the birds in this shed, due to differences in gut microbiota reported between older and younger birds [36]. Each farm is a unique ecosystem with distinct bird genetics, feed, management and environmental conditions, all of which shape microbial diversity. Some factors that may influence differences in alpha diversity include the type of production, breed and age of the birds, as each will have distinct microbiomes. Broilers, layers and breeders have different physiological needs and gut microbiomes. Each farm often uses different feed formulations based on cost, growth goals or health strategies. Variability in diet, such as corn versus wheat-based feed, the feed's protein content, and the use of different FAs, including pre- and probiotics, will influence the manure microbiome and subsequently influence alpha diversity. Farm management practices such as litter reuse and stocking density can increase stress and pathogen loads, shifting the bacterial community structure and affecting alpha diversity [37].

Manure management practices, such as the length of time the manure is stored for and whether it is composted, dried, or piled, change the microbial community and the abundance of aerobic versus anaerobic bacteria. Reused litter accumulates bacteria, including pathogens such as *E. coli*, fungi and antibiotic resistance genes (ARGs) over time [38]. Each reuse cycle shifts the microbial baseline, making the community more diverse or pathogen-heavy depending on management. Environmental factors such as temperature and humidity may also impact microbial survival and growth. The use of different local soil or water sources can introduce distinct environmental bacteria. Biosecurity practices employed by the farms may also be an important factor in the alpha and beta diversity, as farms with poor biosecurity may result in the litter microbiome being influenced by external microbial contamination. Alpha diversity metrics are a valuable tool for evaluating the richness of the microbial community in each sample; however, they do not account for the differences in taxonomic composition between farms.

Several genera were detected in this study that are associated with the degradation of the test compounds. This includes *Pseudomonas* spp. which have been associated with the degradation of trimethoprim, sulfadiazine and salinomycin [32], [39]. Farm C had a more elevated relative abundance (see Supplementary Information Fig. S1 in Appendix D) of *Pseudomonas* than the other two farms. This may have contributed to the faster degradation of salinomycin and trimethoprim in Farm C litter. *Bacillus subtilis*, *E. coli* and *Staphylococcus aureus* are reported to be involved in the biodegradation of trimethoprim [32]. The biodegradation of monensin has been associated with *Stenotrophomonas mataphilia* [40] in contrast to salinomycin degradation, which is associated with *Enterobacter agglomerans* [39]. However, there is limited data on the specific microbial degradation pathways involved, particularly for test compounds not used in human medicine, such as tiamulin, or those regulated as FAs, such as monensin and salinomycin. Nguyen et al., [41] reported a correlation between the abundance of *Achromobacter, Delftia, Flavobacterium, Pseudomonas* and *Stenotrophomonas* and the degradation of tiamulin; however, the mechanisms and species involved are largely unknown. The relative abundance of *Flavobacterium* and *Pseudomonas* in Farm C samples may have influenced the faster degradation of tiamulin in Farm C samples (see Fig. 1d). Several genera where resistance to the test compounds has been previously observed were also detected in the samples. Sulfadiazine is a sulfonamide antibiotic that inhibits dihydropteroate synthase (DHPS) in the folate synthesis pathway. Common Gram-negative carriers of sulfonamide resistance include *E. coli*, *Citrobacter* spp., *Acinetobacter baumannii*, and *Shigella* spp. [42]. In this study, *Acinetobacter* was detected in samples from Farms B and C. Among Gram-positive bacteria, *Enterococcus* spp., were identified by metabarcoding, and in all study samples by culture-based methods.

This variation in relative abundance, resistance mechanisms and ARGs is important as these microbes have a wide range of functions that may impact the fate and behaviour of VMPs and FAs in poultry litter.

4.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *Enterococcus* isolates revealed significant differences in the zones of clearance around wells containing tiamulin across the different farms, as shown in Fig. 5. However, the authors recognise the limitation of a sample size of n = 2 in drawing robust conclusions. Several resistance mechanisms may be responsible, such as ribosomal modification caused by mutations in the 23S rRNA genes, which reduce tiamulin binding affinity or through modification of ribosomal proteins L3 or L4, which affects the binding sites [43]. Efflux pumps are another potential resistance mechanism, and active efflux systems can pump tiamulin out of the bacterial cell, which lowers intracellular concentrations [44]. Enterococci can also acquire resistance genes via plasmids or transposons, which contribute to multidrug resistance profiles; however, this is not well documented in the specific case of *Enterococcus* resistance to tiamulin. Tiamulin is rarely used directly against *Enterococcus* infections; however, resistance monitoring is crucial in veterinary settings where tiamulin is widely applied. There is concern over ARG transfer from animal *Enterococcus* strains to human pathogens. Resistance can reduce the efficacy of pleuromutilins, and whilst tiamulin is not used in human medicine directly, this may become an issue if usage of novel human-use pleuromutilins such as Lefamulin increases [45].

When comparing the zones of clearance on day 1 versus day 61 for each isolate-test compound combination, the zones of clearance for sulfadiazine increased, indicating an increased effectiveness over time (Fig. 5e). This observation is particularly notable, as the efficacy of the other antimicrobials remained relatively stable, highlighting a distinct temporal response to sulfadiazine among Farm C isolates. When considering the degradation data, sulfadiazine degraded rapidly in litter from Farm C (DT_{50} = 1.44 days), suggesting the isolates became less resistant over time. *Enterococcus* spp. often carry resistance genes on mobile genetic elements such as plasmids or transposons. Due to the lack of selective pressure or fitness cost, the isolates may have lost these genetic elements over time, increasing the susceptibility of the isolates to sulfadiazine. Interestingly, these changes were not observed in isolates from the other two farms, as sulfadiazine also degraded very rapidly in these litters. This phenomenon may be attributed to a population shift within the Farm C samples, whereby a more susceptible subpopulation outcompeted the resistant population by day 61. Further investigation, including screening for *sul* genes, may clarify whether the

observed differences are due to a loss of resistance in Farm C isolates, potentially due to plasmid loss or gene dropout, or a more susceptible population was present from the outset but went undetected on day 0 due to sampling limitations. It would also determine whether resistance is linked to mutational activation of the gene or altered expression of *sul* genes, or if resistance persists in the absence of *sul* genes, suggesting the involvement of alternative mechanisms such as efflux pumps. These possibilities could be investigated using PCR, quantitative PCR or whole genome sequencing.

The increased inhibition zone diameter in isolates from Farm B litter samples on day 61 which is observed for salinomycin (Fig. 5a) and tiamulin (Fig. 5d) may be a result of the bacteria acquiring resistance due to the presence of low levels of the antibiotics being present in the litter over the 61 days.

Although AMR is an increasingly important global concern, it is not currently included in the ERA of VMPs or FAs. Most AMR research focuses on antimicrobials used exclusively in human medicine. However, there is growing concern about the effects of veterinary antimicrobials, particularly regarding how resistance to these substances may impact animal and environmental health, contribute to co-selection and ultimately affect human health. The results of this study suggest that poultry litter serves as a reservoir for potentially resistant bacteria, although the clinical relevance of this resistance remains uncertain due to the absence of established breakpoints for *Enterococcus* isolates. Further research is needed to establish breakpoints, epidemiological cut-off values and minimum inhibitory concentrations to accurately assess the risk posed by veterinary antimicrobials in the environment. Continued surveillance of AMR within the poultry production environment is also critical in understanding the dissemination of ARGs and determining whether AMR should be considered in the ERAs for these compounds.

5. Conclusion

This research underscores the necessity of using multiple litter types from diverse sources in manure degradation studies within ERAs. The variability observed in the degradation kinetics of the five test compounds further emphasises the need for additional investigation into the environmental behaviour of other VMPs and FAs to comprehensively evaluate their potential environmental risks. The OECD 307 guideline for soil degradation studies mandates the use of four distinct soil types to account for variability between soils [46]. Findings from this research suggest that degradation studies in manure should similarly incorporate multiple litter types to adequately capture environmental variation. The fate and behaviour of VMPs and FAs in poultry litter remains relatively understudied. Additional research is required to enhance our understanding of this complex matrix and to refine existing risk assessment guidelines. Future studies should focus on identifying the factors driving variability among poultry litters, which may include bedding material, physicochemical properties such as pH, microbial community composition, and the presence of other co-contaminants within the litter.

CRediT authorship contribution statement

Beth Adams: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chris Sinclair:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Edward Haynes:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Paul Kay:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Philip Rooney:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Victoria Pratt:** Writing – review & editing, Supervision, Resources, Project administration. **Laura Carter:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Author note

Chris Sinclair and Philip Rooney are no longer at the Centre for Chemical Safety and Stewardship, Fera Science Ltd.

The authors have no conflicts of interest to declare.

The authors confirm that the data supporting the findings of the present study are available within the manuscript and supporting information. Raw sequencing data is available from NCBI (accession: PRJNA1347738).

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2026.102895.

Data availability

Authors confirm that data supporting the findings of the present study are available within the manuscript and supporting information. Raw sequencing data available from NCBI accession: PRJNA1347738.

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