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Coccidiosis prevention strategies shape the microbiome, resistome and mobilome composition in the broiler gut

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Abstract

Background Coccidiosis is a parasitic infection in the gut of livestock that poses a significant health challenge in poultry farming, underscoring the important role of intervention and prevention strategies in the poultry industry. The use of anticoccidial drugs raises concerns about antimicrobial resistance (AMR) due to their antimicrobial properties and the ability of bacteria to evolve resistance to these drugs. Whether anticoccidial drug resistance could extend beyond coccidiostats, leading to cross-resistance and co-selection against other antimicrobial resistance genes (ARGs), is currently under discussion. Also, it is not well understood to what extent coccidiosis reduction strategies may enable the emergence of ARGs in farm environments and transmission of ARGs to other environments through bacterial clonal transfer or horizontal transmission via mobile genetic elements (MGEs) like plasmids or transposons.

Results In this study, we used metagenomic sequencing of caecal and faecal dropping samples from broiler chickens to investigate how two anticoccidial prevention strategies (vaccination and coccidiostat drugs) influence bacterial taxonomic composition and ARG profiles. We also explored the mobile resistome, ARGs located on mobile genetic elements (MGEs) such as plasmids, which are capable of disseminating, investigating ARGs identifying with the potential to disseminate within and beyond farm settings. Our exploratory findings in bacterial composition, as well as resistome composition with 21 differentially abundant ARGs, illustrating the potential impact of anticoccidial strategies on the chicken gut microbiome and resistome. We also identified 14 plasmid fragments containing ARGs in faecal dropping samples, highlighting mobile ARGs potentially able to disseminate to other environments, including humans.

Conclusions Our findings demonstrate the impact of anticoccidial strategies on the chicken gut microbiome and resistome with potential consequences for the dissemination of ARGs.

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Background

Among all farmed animals, chickens represent the most abundant and most consumed livestock globally. Poultry meat production is projected to contribute nearly half of the total increase in meat production by 2034 [1]. Coccidiosis, an infection caused by protozoan parasites, most commonly from the genus *Eimeria (i.e.*, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox* and *E. tenella*), is one of the most frequent health issues in poultry farming [2]. Depending on the causative agent, the infection can cause effects from intestinal lesions to impaired feed conversion, subsequently leading to stunted growth, or, due to wet litter footpad dermatitis, severe illness and mortality [2]. The worldwide financial impact of the disease is estimated at around 2 billion euros per year [3]. Coccidiosis prevention strategies are therefore of high importance in the poultry industry, and the sustainability of the current poultry production in Europe depends on the effectiveness of the anticoccidial control program [4]. Coccidiostats, anticoccidial medicines, are commonly administered as feed additives in broiler chickens [5]. These coccidiostat drugs are composed of ionophores, which possess the ability to create pores in the membrane of the unicellular parasite, leading to cell inactivation and death, and synthetic molecules, like decoquinate, diclazuril, halofuginone, robenidine, and nicarbazin, that interfere with the eimerial life cycle through other mechanisms [6]. To date, although the onset of resistance can be slowed by using rotation programs [7], anticoccidial resistance has been documented for all the commonly employed anticoccidial drugs [2], posing a significant challenge to effective disease control and highlighting the urgency for alternative or diversified prevention strategies.

In addition to anticoccidial activities, ionophores also possess antimicrobial activity in vitro, and bacteria are able to develop resistance toward these molecules [8]. Additionally, resistance to ionophores has been linked to increases in resistance to other antimicrobials. Specifically, cross-resistance has been reported between bacitracin and monensin, and an ABC transporter causing resistance to narasin, salinomycin, and maduramicin has been found encoded on a plasmid that also contained the *vanA* gene cluster, indicating possible co-selection of these resistance genes [9–11]. Antimicrobial resistance genes that emerge and are selected for in farm environments can be transmitted to humans via direct animal-human contact, the food chain, or via animal-human shared environments [12]. Dissemination of resistance may not only occur as a result of bacterial clonal transmission between animal and human domains but also via horizontal transmission of antimicrobial resistance genes (ARGs) encoded on mobile genetic elements (MGEs) such as plasmids or transposons [13, 14]. This increases the scale at which ARGs can be disseminated, asking for close monitoring of resistance in bacteria not directly related to human health.

Because of their antimicrobial activity, ionophores are prohibited under the NAE (No Antibiotics Ever) and RWA (Raised Without Antibiotics) programs in the United States [15], even though they are not classified as antibiotics by the FDA. Outside of these programs, however, coccidiostats remain widely used in broiler production. In the European Union, ionophores are legally classified as coccidiostats rather than antibiotics. This regulatory distinction has historically allowed poultry treated with ionophores to be marketed as "antibiotic-free," despite their antimicrobial properties. As an alternative, vaccines have been developed that enhance immune activation in the host, leading to a completely protective immune response, but that can also cause mild damage to the intestinal epithelium due to the transient parasitic replication in the gut during vaccine-induced immunity [16–19] Despite the importance of coccidiostats and vaccination against coccidiosis in animal health care, not much is known about how these two different strategies influence the microbiome and resistome composition in broilers. Furthermore, to what extent resistance genes selected in the chicken gut microbiome can be mobilised and persist outside the gut, posing a risk of environmental transmission, is largely unknown. This is relevant because substantial efforts to decrease antimicrobial usage and enforce regulations to prevent antimicrobial resistance (AMR) in poultry farms have been made, such as restricting antimicrobial use exclusively to veterinary purposes in Europe and the United States. Although these measurements have been shown to decrease the abundance of AMR [20], epidemiological studies consistently reveal the persistent presence of AMR in broiler farms across the world [21, 22], and ARGs can persist in the gut environment long after cessation of antimicrobial use [23]. Therefore, it is essential to systematically assess the impact of all aspects of poultry health management and development in the context of AMR surveillance and control.

Our study builds upon existing knowledge by employing an integrated approach, combining shotgun metagenomic sequencing with a novel targeted resistance gene capture platform (ResCap). This methodology offers unparalleled sensitivity for detecting a wide array of antimicrobial resistance genes, particularly low-abundance mobile ARGs, which significantly enhances our ability to characterize the broiler gut resistome and mobilome in unprecedented detail. This combined approach represents a key methodological advantage and a novel contribution to understanding AMR dynamics in poultry.

The aim of this exploratory study was to (i) systematically characterize the bacterial taxonomic composition,

the total resistome, and specifically the plasmid-borne mobile antimicrobial resistance genes (mARGs) in broiler chickens, investigating their potential for dissemination; (ii) compare the gut microbiome and resistome profiles between broilers raised under two distinct anticoccidial prevention strategies: coccidiostat administration and vaccination; (iii) assess the relationship between microbial community composition and resistome profiles; and (iv) evaluate the differences in resistome and mARG profiles in caecal and faecal dropping samples, examining their implications for environmental AMR surveillance and transmission.

Methods

Animals and experimental design

160 samples were collected for this study from a Dutch poultry farm located at the German border (49824 Ringe, Germany). The farm was chosen based on general high consistency of animal performance and low mortality to provide a stable and well-controlled background for the experimental interventions. The farm adhered to standard commercial broiler management practices, including thorough cleaning and disinfection protocols between production rounds, aiming to minimize environmental carry-over and biosecurity risks. The study focused on 21-day-old broiler chickens (Ross 308), all floor-raised from the same hatchery and same genetic background. This age point was strategically selected as a critical early-life intervention phase, allowing sufficient time for both coccidiosis prevention strategies (coccidiostat administration and vaccination) to exert their effects and shape the developing gut microbiome and resistome, thereby enabling the assessment of their initial impact on the broiler gut ecosystem. Two consecutive flocks (production rounds) hosted in the same poultry house were analysed. The first flock received a combination of 50% nicarbazin and 50% narasin (Maxiban, Elanco US), while the other flock underwent oral vaccination using Paracox-5 (MSD Animal Health). Both groups were provided with the same wheat-based start feed (MatchFeed 1, Agrifirm) for the initial 10 days, followed by a soy- and wheat-based grower feed (MatchFeed 2, Agrifirm) up to the 21st day. No antibiotics were administered throughout the study. Specific environmental conditions (e.g., temperature, humidity) were not continuously monitored for this study, though standard climate control practices for broiler houses were maintained. 50 broilers were randomly selected for each intervention and cervical dislocation was performed following EU regulations for euthanasia. The caecum was then extracted after abdomen incision, and the caecal content was collected in sterile 2mL eppendorf tubes. Concurrently, 30 fresh-looking faecal droppings were gathered from the ground for each intervention at various locations within the house (carefully avoiding contact with bedding material to minimize environmental contamination). After transportation (a two-hour period at room temperature), all samples were initially aliquoted and subsequently stored at -80 °C.

DNA extraction and sequencing

After thawing of individual aliquots, DNA extraction was performed following a modified version of the QIAmp Fast DNA stool mini kit (Qiagen, Venlo, The Netherlands) protocol [24]. 0.2 g of faecal material, 500 µl of 0.1 mm zirconium beads (Lab services), and 1 ml of InhibitEx buffer (Qiagen) were combined in a 2 ml Sarstedt tube. Two cycles of bead-beating were performed at 3800 rpm for two minutes each, with a two-minute pause on ice in between, using a Mini-beadbeater-24 (Biospec, Rijswijk, the Netherlands). These parameters were optimized to ensure efficient cell lysis and DNA extraction from complex faecal samples while minimizing excessive DNA shearing. The mixture was incubated at 95 °C for 7 min and centrifuged at 16,000 x g for 1 min. The resulting supernatant was collected (400 µl) and treated with proteinase K, followed by incubation at 70 °C for 5 min. The mixture was passed through a spin column following the DNA stool mini kit protocol (Qiagen). The DNA was eluted using EB elution buffer (Qiagen) in DNA LoBind microcentrifuge Eppendorf tubes (VWR International, Amsterdam, the Netherlands) and quantified using the Qubit 2.0 fluorometer (Invitrogen). The KAPA Hyperplus Kit (Roche), along with the KAPA Universal UMI Adapter (cat number 9329862001) in conjunction with KAPA UDI Primer Mixes (cat numbers 9329838001 and 9329846001), was utilized to prepare DNA libraries following the KAPA HyperCap Workflow v 3.2 (Roche) instructions. The caeca samples were treated individually. For the faecal dropping samples, six libraries, each composed of five equimolar, individually extracted samples, were constructed. This pooling strategy was employed because the faecal droppings were collected from various locations on the floor, representing a community-level snapshot of the environmental resistome and mobilome present within the poultry house, rather than attempting to capture individual animal-specific gut profiles. All libraries were assessed for DNA concentration and fragment size using the Agilent Bioanalyzer DNA High Sensitivity kit (2100 Bioanalyzer), and adapter ligation efficiency was measured using the KAPA Library Quantification Kit (Roche). Subsequently, the libraries were multiplexed and sequenced on the NovaSeq 6000 platform (Illumina), employing 2 × 150-bp paired-end sequencing per flow cell. As an experimental internal control, a negative control (no sample) and a positive control (mock community standard Zymobiomics D6300) were included in the extraction and sequenced. A probe-based

resistance gene capture platform, ResCap (Roche), was used on 60 caeca and all 12 pooled faecal dropping samples. ResCap offers enhanced sensitivity for detecting a wide array of antimicrobial resistance genes by specifically enriching target sequences through probe hybridization, thereby complementing shotgun metagenomics by identifying potentially low-abundance but clinically relevant ARGs that might otherwise be missed. Briefly, as described in the original paper [25], the 72 individual purified libraries were pooled in six groups of 12 in equimolar ratio to obtain 1.2 µg of total library per pool. After pooling, each sample was incubated with the KAPA HyperCap target enrichment probes (Roche) for 36 h at 37 °C to perform probe hybridization. Subsequently, the enriched DNA samples were precipitated using biotin-labeled beads (capture beads, Roche). Next, on-beads amplification of the enriched DNA was performed on captured DNA using PCR. After amplicon DNA purification, DNA concentration and fragment size of the obtained libraries were measured using the Agilent Bioanalyzer DNA High Sensitivity kit (2100 bioanalyzer).

Bioinformatic analyses

Sequence quality control

To ensure the quality of the sequencing reads, the reads were subjected to a quality control step. First, fastp [26] was used for UMI (Unique Molecular Identifier) extraction (--umi/-U --umi_loc = read1 --umi_len = 8) and deduplication (--dedup/-D), as well as general quality control. Subsequently, the deduplicated reads were used as input for the quality control module of MetagenomeAtlas [27], integrated into the MetaMobilePicker pipeline [28]. Briefly, this step performs further adapter removal, read quality filtering, and host DNA removal. This last step was performed using the GRCg6a Gallus gallus reference genome (GCA_000002315). The deduplicated reads were employed as input for the quality control module of MetaMobilePicker. Samples with fewer than 10 million reads or more than 200 million reads after quality control were excluded. Analysis was also performed on negative control (no sample) and a positive control (mock community standard Zymobiomics D6300).

Taxonomic analyses

R version 4.2.2 was used for all statistical analyses. To construct a taxonomic profile for each sample, Metaphlan 4.0.3 [29] was employed, using its default parameters. These profiles were subsequently combined by using the merge_metaphlan_tables script within the metaphlan tool.To remove biases caused by differences in sequencing depth in all downstream analyses, the count table was rarefied using the Phyloseq package's rarefy_even_depth function [30], standardised to the depth of the sample with the lowest counts (10,629,520 counts). This rarefied count table was used for alpha and beta diversity calculations. Alpha diversity metrics, including Chao1, the Shannon index, and the inverse Simpson index, were calculated using the Microbiome package. Significance testing of these metrics was conducted through a two-tailed Wilcoxon Rank Sum test, adjusting for multiple comparisons using the Bonferroni correction method.

To assess dissimilarities in beta diversity, the Phyloseq package's ordinate function was employed to create Principal Coordinates Analysis (PCoA) plots for faecal droppings and caecal samples separately. PCoA plots were generated using Bray-Curtis dissimilarity metrics. To analyse centroid distances, PERMANOVA test was used on the Bray-Curtis distance matrices using the adonis2 function from the vegan package [31] with 10,000 permutations.

To detect differences in dispersion between intervention groups within the PCoA space, the betadisper function as part of the vegan package was employed and the significance of these dispersion differences was tested via a permutation test, using the default parameter of 10,000 permutations.For the identification of differentially abundant genera per intervention group in the caecal samples, the ancombc2 algorithm, integrated within the ANCOMBC R package [32], was used. This analysis was conducted specifically on the caecal samples. To adjust p-values for potential false discoveries, the false discovery rate (FDR) method was applied. Furthermore, the neg_lb parameter was used to detect and classify structural zeroes, while other parameters remained at their default settings.

Resistome composition

To explore the reservoir of ARGs within the metagenomic samples, quality-controlled reads were aligned to the ResFinder database [33] using the kma aligner [34], using parameters – 1t1 and -cge to facilitate one-to-one mapping and the use of the CGE scoring matrix. In order to aggregate the genes featuring numerous alleles in the ResFinder database and reduce the number of zero-counts in ARG clusters, CD-HIT EST [35] was used, using all ResFinder sequences and an identity threshold of 90% and a length threshold of 90%, and all counts for each cluster were summed. These ARG clusters will be referred to as resistance genes.

To add metadata on ARGs and ARG classes, the ARG metadata file generated in a prior study was utilized and genes that were added to the ResFinder database since the publication of this study were included [36]. Missing genes were first matched on gene prefixes in this previously published dataset and remaining gaps were resolved via manual inspection and information from the CARD database [37].

Two count matrices were constructed by summing the count values per gene and AMR class. These will be referred to as the gene matrix and class matrix.

For visualisation purposes, Fragments per Kilobase Million (FPKM) were computed based on the total number of bacterial reads in each sample, accounting for variations in sequencing depth, abundance of non-bacterial reads, and gene length among the ResFinder genes.

The inference of differential abundance for ARGs across interventions was done using DESeq2 on the unnormalized count matrix [38]. To address zero-count-related issues, the poscount method for size factor estimation was employed. Furthermore, ARGs in low abundance, defined as being present in less than ten samples with a cut-off of three fragments, were excluded. The detection of differential abundance was done using a two-sided Wald test ($\alpha < 0.05$).

This analysis was performed on the gene and class-level count matrices. Using the same procedure as for the taxonomic counts, rarefying and alpha diversity estimation of the poultry resistome were performed. Using the Microbiome package [39], alpha diversity metrics chao1, Shannon index, and inverse Simpson index were calculated using the rarefied count table. Significance testing of these metrics was done through a two-tailed Wilcoxon Rank Sum test, with adjustment for multiple comparisons using the Bonferroni correction method.

To assess dissimilarities in beta diversity, the Phyloseq package's ordinate function was employed to create Principal Coordinates Analysis (PCoA) plots for faecal droppings and caecal samples separately. The PCoA plots were generated using Bray-Curtis dissimilarity metrics. To analyse centroid distances, the PERMANOVA test, using the Bray-Curtis distance matrices, was employed with the adonis2 function from the Vegan package, with 10,000 permutations.

To detect differences in dispersion between interventions within the PCoA space, the betadisper function as part of the vegan package was employed. The significance of these dispersion differences was determined via a permutation test, with 10,000 permutations.

Procrustes analysis

To assess potential correlations between the taxonomic composition and the resistome, a Procrustes analysis was conducted utilising the procrustes function within the ade4 package. Prior to analysis, Hellinger transformation was performed on the rarefied count matrix representing genus-level taxonomic data and the gene-level resistome dataset to transform absolute to relative values, using the decostand function as part of the vegan package. Subsequently, ordination was performed using Phyloseq's ordinate function, specifically using the Principal Coordinates Analysis (PCoA) method on Bray Curtis distances. For permutation testing, the procruste.randtest function of the ade4 package was used with 10,000 permutations.

Mobilome composition

To establish associations between ARGs and MGEs, the samples were analysed with the MetaMobilePicker pipeline [28]. This pipeline specifically assembles metagenomic reads into contigs, identifies mobile genetic elements (MGEs), and determines if ARGs are co-localized on these elements. Contigs larger than 2 kb, annotated with mobile elements and containing ARGs, were selected and combined across all samples. Subsequently, clustering was performed using mmseqs2 [40], with coverage mode 0 and an identity and length threshold of 90%. Metagenomic reads from all samples were uniquely mapped to the cluster representatives via Bowtie2 [41], using default settings. Additionally, to aid in the downstream analyses, Anvi'o [42] was employed to create profile databases for each sample, which were merged using Anvi-merge.

To provide a comprehensive overview of the abundance of each MGE, fragments per kilobase million (FPKM) values were calculated by analysing reads mapped to plasmid and phage-related clusters. In the case of insertion sequences (IS), bedtools [43] was used to extract reads specifically aligning with IS regions.

To investigate the mobile fraction of resistance (mARGs), only the MGE clusters harbouring ARGs were included in the post-analysis. Using Anvi'o, detection values (breadth of coverage) were quantified for each mARG. An mARG cluster was deemed present in a sample when the detection value exceeded 0.9. Based on presence patterns, mARG clusters were separated into three categories: faecal droppings-specific, caeca-specific and general clusters. Clusters were attributed to a category based on their presence in 90% of samples. In case the cluster was present in at least 90% of all samples, it was attributed to the general category.

mARG clusters were grouped based on shared ARGs for visualisation using the ARGs flanking regions. Using Pangraph and a modified version of the flanking-region pipeline of Shaw et al. (2023) [44], grouped mARG clusters were visualized based on shared ARGs using the ARGs flanking regions. The pipeline was modified to include contigs with short and imbalanced flanking regions and not discard contigs with no additional genes. Using the vega editor (https://vega.github.io/editor/#/edited), the resulting image files were edited to centre the ARG. Groups of genes present in more than one cluster were further investigated using BLAST against the nucleotide (nt [45]) and RefSeq [46] databases to further annotate the genomic context of these mARGs, using default parameters.

ResCap profiling

ResCap reads were submitted to quality control and deduplication using fastp. Using the kma aligner, high-quality reads were aligned to the ResFinder database using parameters –1t1 and -cge to facilitate one-to-one mapping and the use of the CGE scoring matrix. Genes were considered present with an abundance of more than 1 fragment per kilobase of gene.

Results

Bioinformatics preprocessing

After sequencing (Fig. 1A), reads of all 112 poultry samples, consisting of 50 caecal samples per intervention (coccidiostat or vaccination) and six pools of five faecal dropping samples per intervention, were used for quality control (QC). Five samples, all originating from caeca (four vaccination and one coccidiostat intervention sample), were excluded from further analysis.

After QC, the 107 samples remaining had an average of 34.8 ± 15.6 million reads for the 95 caecal samples and 33.7 ± 15.6 million reads for the 12 pooled faecal dropping samples. The reads remaining after each QC step are shown in Supplementary Table 1. On average, 30.8% ± 7.1% of reads originated from bacteria as determined by taxonomic analysis. To ensure data integrity, a negative control yielded negligible reads (< 100) with no detectable taxa or ARGs, confirming minimal contamination. The Zymobiomics D6300 mock community's taxonomic profile (Figure S1) accurately matched its known composition, validating our pipeline for unbiased microbial and ARG detection.

Distinct diversity and similar richness for intervention types

The alpha diversity showed no differences between interventions (Fig. 1B and Figure S2A). The alpha diversity metrics in the faecal droppings were notably lower than those in the caeca samples; however, the two different sample types cannot be directly compared due to the different nature of the samples. Two-tailed Kruskal-Wallis tests showed no significant differences between intervention types after Bonferroni correction at an adjusted p-value of 0.05.

To infer beta diversity as a metric for between-sample diversity, we ordinated the rarefied count data using Principal Coordinates Analyses (PCoA, Fig. 1C). These PCoA ordinations showed distinct clusters for intervention types in both sample sources. PERMANOVA analysis comparing intervention types showed significant differences in intervention types in bacterial taxonomy between intervention types (caeca: $R^2 = 0.092$, $F = 9.47$, $p < 0.001$; faecal droppings: $R^2 = 0.30$, $F = 4.32$, $p < 0.05$). The dispersion of the points in the PCoA was not found to be significantly different between intervention types ($p > 0.05$), suggesting no significant difference in the variance of the beta dispersion in each group.

Coccidiostat and vaccination have different effects on the taxonomic composition

To investigate differences in taxonomic composition between the two anticoccidial intervention types, coccidiostat and vaccination, we first investigated the relative abundance at the phylum level (Figure S3) and calculated the ratio of *Bacillota* (formerly *Firmicutes)* to *Bacteroidota (*formerly *Bacteroidetes)*. On average, this ratio was higher in the vaccination (mean = 24.5 ± 30.1) than in coccidiostat samples (mean = 13.6 ± 27.5) (Figure S4), indicating a shift in the relative abundance of these phyla. Next, the relative abundance of each of the taxa in the rarefied taxonomic counts dataset was calculated and the ten most abundant genera per sample type were identified (Fig. 1D). This shows a largely similar pattern in relative abundance of bacterial genera in the caecal samples. The distribution of genera in the faecal droppings differed considerably from that in the caecal samples. The faecal dropping samples were largely composed of the *Lactobacillus* genus (mean = 52.9% ± 18.0%, range: 22.4% to 76.6%), with a higher abundance in the vaccinated samples (mean = 63.8% ± 18.3%) than in the coccidiostat faecal dropping samples (mean = 42.0% ± 16.2%). For the genus *Escherichia*, a higher relative abundance was observed in the coccidiostat samples (mean = 28.4% ± 22.0%) compared to vaccinated samples (mean = 9.6% ± 14.1%).

To determine taxa in differential abundance between intervention types, ANCOM-BC2 on the unrarefied caecal dataset at the genus level was used (Fig. 1E). This resulted in a total of 21 genera being overabundant in the vaccination samples, and 11 genera being overabundant in the coccidiostat samples. Differentially abundant genera and their higher taxonomic ranks are shown in Supplementary Table 2. For 15 of the 32 differentially abundant taxa it was possible to define only a higher taxonomic rank, while the other taxa remaining 17 were assigned to a known genus name. The genera, *Gemmiger*, *Anaerotignum*, *Butyricimonas*, *Butyricicoccus*, *Faecalibacterium and Lactobacillus* were more highly abundant in vaccination samples compared to the coccidiostat samples while *Streptococcus*, *Bifidobacterium*, *Candidatus Pseudoruminococcus*, *Candidatus Neoclostridium and Alistipes* had higher relative abundance in the coccidiostat group compared to the vaccination group. Based on these analyses, we concluded that coccidiostat intervention and vaccination had a different impact on the taxonomic composition of the broiler microbiome.

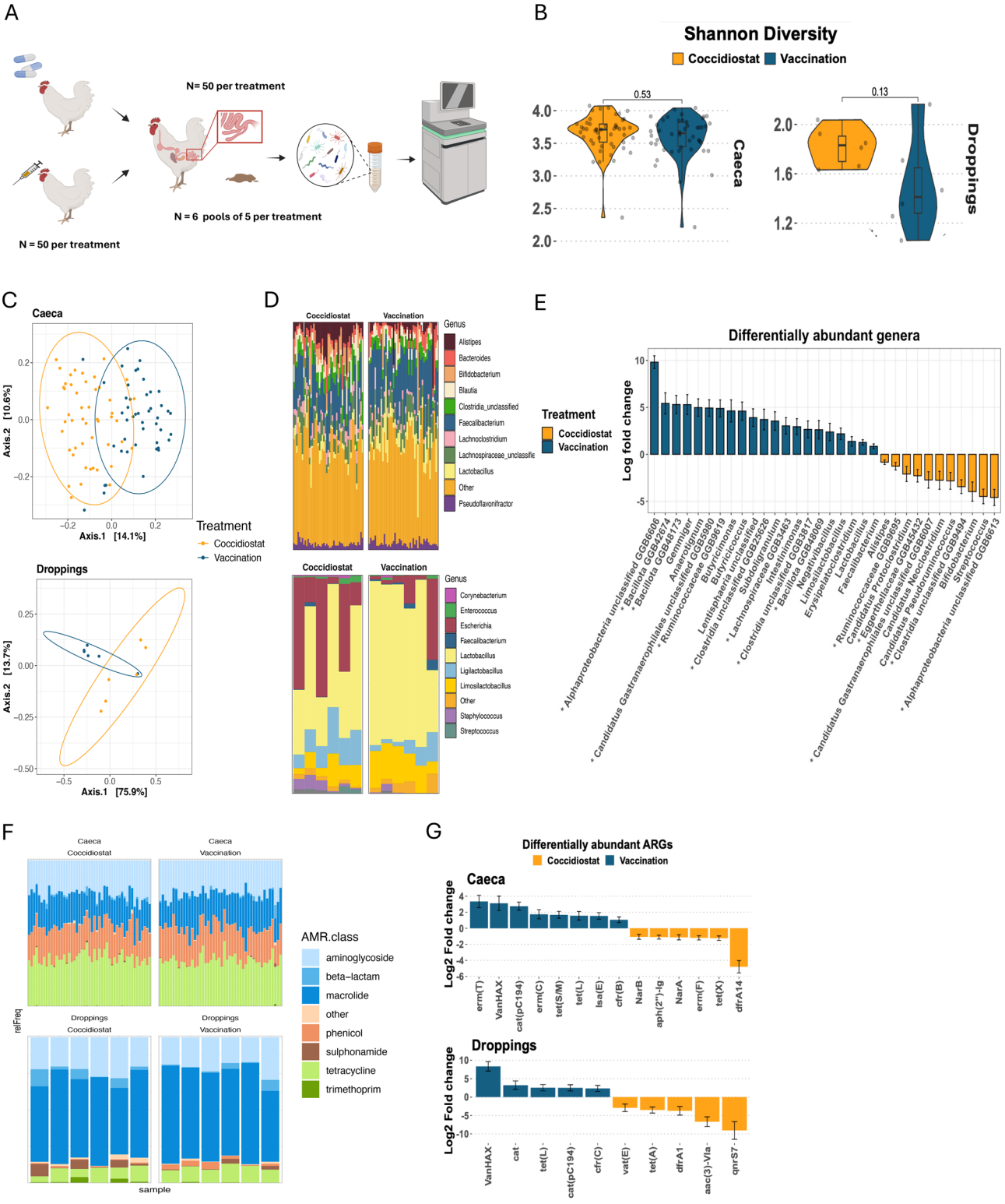

Fig. 1 (**A**) Experimental setup (**B**) Within-sample taxonomic diversity per sample type and per treatment. (**C**) Between-sample taxonomic diversity ordination (**D**) Relative taxonomic composition on genus level (**E**) Differentially abundant genera in caecal samples. Asterisks indicate that the genus was given a name based on a higher taxonomic rank. (**F**) Relative resistome composition per class of antimicrobial resistance. (**G**) Differentially abundant antimicrobial resistance genes (ARGs) and their log fold-difference

Similar abundance but varying composition of ARGs between interventions

In order to investigate the differences in resistome composition between interventions, we compared the abundance of ARGs found in the caecal samples and faecal droppings of coccidiostat-treated or vaccinated broilers. For this, we determined the number of mapping read pairs per sample to each ARG of the clustered ResFinder database and calculated bacterial FPKM values for normalisation purposes. The sums of these FPKM values per sample are shown in Figure S5. We calculated the number of distinct ARGs found per intervention and per sample type, resulting in a total of 186 distinct genes, originating from 15 ARG classes, found with at least one fragment per kilobase of gene. Specifically, 164 ARGs were found in the caecal samples, with 141 in the coccidiostat and 131 in the vaccinated samples. In the faecal dropping samples, 146 genes were found, with 127 distinct genes for both coccidiostat and vaccinated samples. Of the 15 ARG classes, the rifampicin class was only found in the dropping samples in both interventions, and the nitroimidazole class was only found in the caecal samples. Additionally, quinolone resistance genes were only found in the coccidiostat samples of both caecal and faecal dropping samples.

No difference in total ARG abundance was found between different interventions; however, a large difference was detected between the FPKM values of the caecal and faecal dropping samples, as the mean FPKM in the faecal dropping samples (mean = 4600 FPKM ± 1112.90 FPKM) was 58.3% higher than the mean FPKM in the caecal samples (mean = 2905 FPKM ± 611.65 FPKM). To get an overview of the total amount of ARG mapping reads, we summed the mapping read counts of each of the 15 resistance classes and calculated the relative proportion of each of these classes (Fig. 1F). This showed a highly similar abundance profile for both interventions, but a highly varying profile between sample types. Faecal droppings had a higher relative abundance of genes encoding for macrolide resistance and a lower relative abundance of genes encoding resistance to tetracycline, phenicol, and aminoglycoside compared to caecal samples.

To determine the within-sample diversity of ARGs, we calculated the Chao1, inverse Simpson and Shannon indices. This revealed no differences in alpha diversity between the interventions in both sample types.

Next, we ordinated the resistome composition to determine between-sample diversity using PCoA. We found a significant difference in the resistome composition of the caecal samples between the coccidiostat-treated and vaccinated broilers (R^2 = 0.023, F = 2.23, p = 0.007). The resistome composition of the faecal droppings was not found to be significantly different between interventions (R^2 = 0.245, F = 3.25, p = 0.044) (Figure S6). Beta dispersion tests indicated no significant differences in within-group variability for either caecal (p = 0.985) or faecal droppings (p = 0.282) samples, supporting the PERMANOVA results.

To determine differentially abundant resistance genes, we used DESeq2 on the ARG class and ARG count matrices (Figs. 1G and 2A and B). Two ARG classes were found to be significantly different between interventions in the caecal samples: narasin resistance was significantly more abundant in the coccidiostat group, while lincosamide resistance was significantly more abundant in the vaccination group (narasin: log fold difference (LFD) = -1.13, adjusted p < 0.001; lincosamide: LFD = 1.29, adjusted p = 0.004) (Supplementary Table 3). Moreover, fourteen ARGs were found to be differentially abundant between intervention groups in the caecal samples. Of these, six genes were more abundant in the coccidiostat group [*aph(2")-Ig*, *dfrA14*, *erm(F)*, *narA*, *narB* and *tet(X)]*, and eight were found more abundant in the vaccination group [*cat(pC194)*, *cfr(B)*, *erm(C)*, *erm(T)*, *tet(L)*, *tet(S/M)*, *lsa(E)* and *vanHAX*] (Supplementary Table 4).

The *vanHAX* operon was found to be differentially abundant, with lower abundance in the coccidiostat group compared to the vaccination group. However, the regularised log values (Fig. 2B) of this gene cluster show a very low abundance of these genes in general, with regularised log values less than 0. Additionally, five macrolide resistance genes were found to be differentially abundant between interventions, where *erm(F)* was more abundant in the coccidiostat group and the other four genes [*erm(C)*, *erm(T)*, *lsa(E)* and *cfr(B)*] were more abundant in the vaccination group. A similar pattern was found for three tetracycline genes that were found to be differentially abundant between interventions. Of these genes, *tet(A)* and *tet(X)* were found more abundantly in the coccidiostat samples, whereas *tet(L)* and *tet(S/M)* were more abundant in vaccination samples (Fig. 1G).

In the faecal droppings, 10 ARGs were found to be differentially abundant, of which three were shared with the caecal samples. Of the differentially abundant genes, five were found more abundant in the coccidiostat group, and seven were more abundant in the vaccination group. At the ARG class level, three classes representing resistance to phenicol, quinolone and trimethoprim were differentially abundant. The differential abundance of these classes were represented by the *cfr(C)*, *cfr(E)*, *cat* and *cat(pC194)* genes conferring resistance to (chloram) phenicol, which were more abundant in the vaccination samples, while the quinolone resistance gene *qnrs7* and the *dfrA1* gene conferring trimethoprim resistance were found more abundant in the coccidiostat samples.

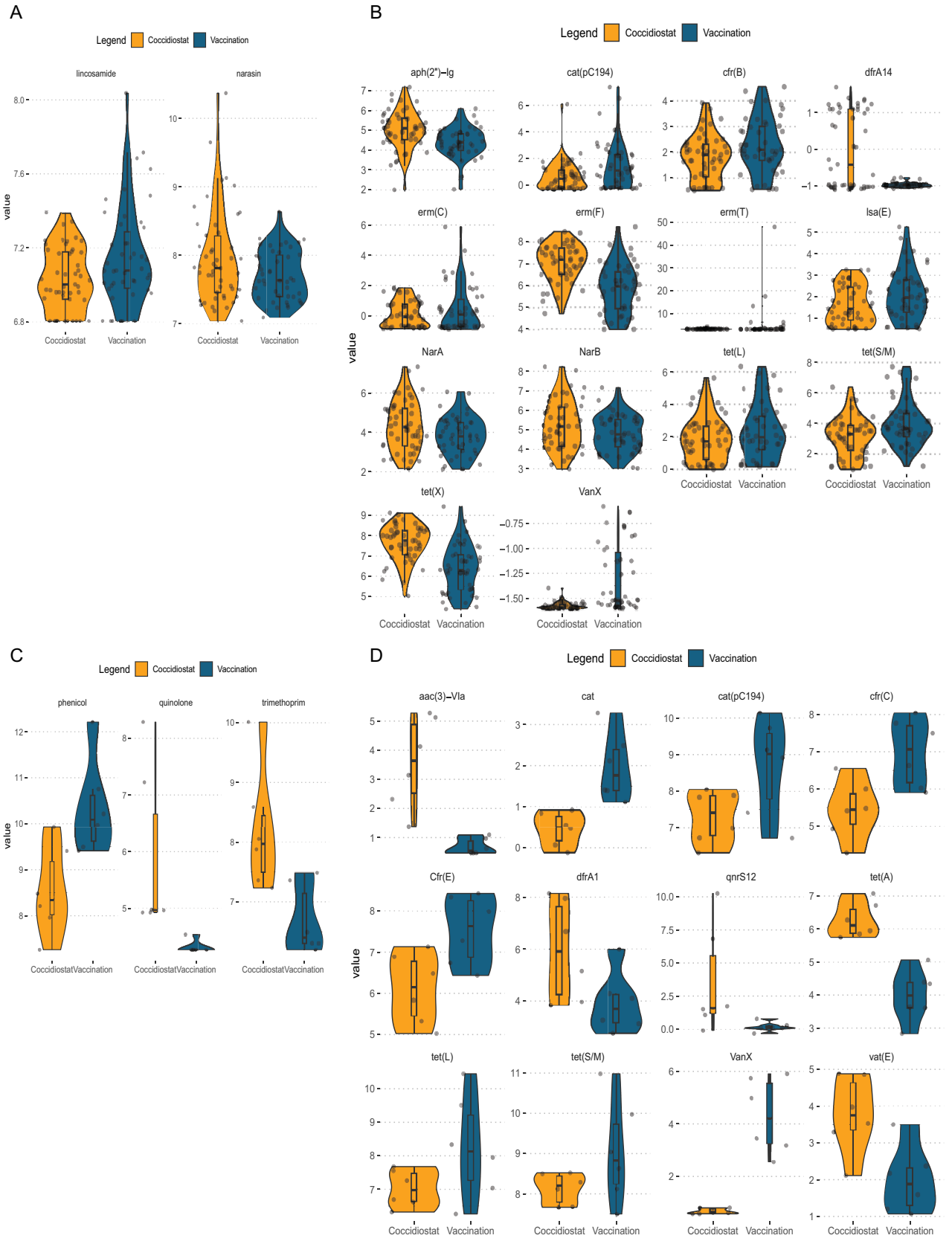

Fig. 2 Regulaized log values for differentially abundant (DA) (**A**) ARG classes in caecal samples. (**B**) DA gene clusters in caecal samples. (**C**) DA ARG classes in faecal droppings. (**D**) DA ARGs genes in faecal droppings samples. Significant at $q < 0.05$, Wald test by Deseq2. Colors denotate treatment type

ResCap confirms differences in resistome composition between preventive interventions

To increase the sensitivity to detect ARGs, we applied ResCap, a resistance capture platform for 7,963 antibiotic resistance genes, on a subset of the caecal samples and all pooled faecal dropping samples. Using ResCap, 249 ARGs were identified with at least one read pair mapped per kb of reference gene in at least one sample.

The ARGs with the largest difference in absolute presence between interventions are shown in Table 1. Most notably, in both sample types, the *qnrS7* gene was found in a majority of coccidiostat samples: 73.9% (17 out of 23) caecal and 100% (six out of six) faecal droppings samples, whereas this gene was not detected in any of the vaccinated samples (0%) (Fisher's exact test, $p < 0.001$ for caecal; $p = 0.002$ for faecal droppings). Conversely, the *vanHAX* gene cluster was found in 78.6% (22 of 28) vaccination caecal samples and 100% (six out of six) vaccinated faecal droppings samples but was not detected in any of the coccidiostat samples in both caeca and faecal droppings (Fisher's exact test, $p < 0.001$ for caecal; $p = 0.002$ for faecal droppings).

In the caecal samples, the *dfrA14* gene was found in 78.3% (18 out of 23) coccidiostat samples and in none of the vaccinated samples (0%) (Fisher's exact test, $p < 0.001$). In the faecal droppings samples, the *mecC2* gene was found in 83.3% (five out of six) coccidiostat samples and in none of the vaccinated samples (0%) (Fisher's exact test, $p = 0.02$).

Of the genes detected by ResCap, 71 were not identified in the metagenomics samples, whereas 33 genes detected in the metagenomics data were not identified in the ResCap data. However, of these 33 genes, only five were present in more than two samples in the metagenomic data. In contrast, the 71 genes only identified in the ResCap data were identified in more than two samples (up to 13 caecal samples and up to 11 faecal dropping samples), indicating that ResCap was able to identify prevalent genes missed by metagenomic sequencing, whereas genes missed by ResCap were found very rarely by metagenomics. Since the ResCap probes were designed with an older version of the Resfinder database than we used for identifying ARGs in the metagenomics samples, we verified whether the 33 genes only found in the metagenomics samples were ARGs not present in the ResCap probes. This analysis showed that 26 genes were indeed not represented by ResCap probes, while even [*aac(3)-VIIIa*, *aac(6')-Iae*, *aph(3')-Vb*, *catB*, *erm(41)*, *fosA7* and *mph(A)*] were present in the ResCap probes, despite not being detected in the ResCap experiment. This could be attributed to a combination of factors, such as potential primer bias or suboptimal hybridization efficiency for these specific probes.

Table 1 Resistance genes detected by ResCap with more than 50% points difference between interventions

Cluster	Sample type	Percentage coccidiostat samples	Percentage vaccination samples
blaVIM-45	Droppings	83.33	33.33
blaZ(cluster 2)	Droppings	83.33	16.67
blaZ(cluster 3)	Droppings	66.67	16.67
dfrA14	Caeca	78.26	0
dfrA5	Droppings	50.00	0
erm(50)	Droppings	100.00	50.00
lnu(E)	Droppings	66.67	16.67
mecC	Droppings	83.33	16.67
mecC2	Droppings	83.33	0
qnrS7	Droppings	100.00	0
qnrS7	Caeca	73.91	0
VanC2XY	Droppings	83.33	33.33
VanHAX	Droppings	0	100.00
VanHAX	Caeca	0	78.57
dfrA17	Droppings	0	66.67
tet(B)	Droppings	33.33	83.33

Different mobile genetic elements in caeca and faecal dropping samples

To get an overview of the ARGs present on MGEs in the metagenomics dataset, we assembled the metagenomic reads into contigs and identified assembled plasmids, phages and insertion sequences (IS elements) (Fig. S7). Figure S7 displays FPKM values of each class of MGE compared between interventions. No difference in the total abundance of plasmid-originating contigs was observed between interventions. Faecal dropping samples had a notably lower plasmid abundance compared to the caecal samples. Phage and IS elements abundance showed no differences between sample or intervention types; however, we found more phages in dropping samples (mean = 25.1 ± 14.2 FPKM) than in the caecal samples (mean = 10.3 ± 10.5 FPKM).

To further assess the abundance of MGEs associated with ARGs and compare between interventions, we investigated the mobile fraction of resistance genes. Briefly, we selected assembled contigs annotated as MGEs and containing ARGs from each metagenome and co-clustered them across all samples. We then scored presence-absence patterns, which facilitated assessing their persistence in passage from the gastrointestinal tract to faecal droppings. This allowed for the identification of mARGs that can contribute to the dissemination of resistance-carrying MGEs.

Predicted mobile ARGs from all samples, focusing on plasmid-predicted ARGs, were clustered together based on sequence identity to define the farm-wide mobile resistome, hereafter referred to as mARG-clusters. To determine the presence of these mARG-clusters in each

sample, detection values (the percentage of bases covered by at least one metagenomic sequencing read) were calculated. In total, 95 plasmid-borne mARG-clusters could be discerned. A heatmap containing the distribution of plasmid-borne mARG-clusters and containing the detection values is displayed in Fig. 3. This heatmap reveals a separation between caecal and faecal dropping samples in the horizontal cladogram. Twenty mARG-clusters could be subdivided into dropping-specific (10 clusters, 4 different ARGs, present in all dropping samples, absent in at least 10% of caecal samples), caeca-specific (6 clusters, 3 different ARGs, present in all caecal samples and absent in at least 10% of faecal dropping samples) and general mARG-clusters (4 clusters, 2 different ARGs, present in all samples). The ARGs in these mARG-clusters are displayed in Table 2, separated by subdivision. All mARG-clusters detected in at least one sample are displayed in Supplementary Table 5.

To further investigate the mobile context of these mARGs in the samples in contact with the outside environment (dropping-specific and general mARG-clusters), we compared the regions flanking each of the ARGs present in more than one mARG-cluster (Fig. 4). Three genes occurred more than once in an mARG-cluster: *tet(W)*, *ant(9)-Ia* and *lnu(A)*, potentially conferring resistance to tetracycline, spectinomycin and lincomycin, respectively.

In total, seven mARG-clusters contained the *lnu(A)* gene (D2, D3, D5-D8 and D10). In five of these, one copy of the *lnu(A)* gene was flanked by an *ant(9)-Ia* gene. In one cluster (D8), two *lnu(A)* genes were present, surrounding an *ant(9)-Ia* gene. Inspection of the *lnu(A)* containing mARG-clusters showed three different contexts in which this gene occurred: flanked by *ant(9)-Ia* (five times), without association with *ant(9)-Ia* (once) and with a duplicated *lnu(A)* gene around *ant(9)-Ia* (once). Comparing the flanking regions outside of the ARGs of the six mARG-clusters with one *lnu(A)* gene showed that each cluster sequence was representing a distinct genetic context. In mARG-clusters D10 and D7, a region of 940 bp upstream of the *lnu(A)* gene was found to be identical. This area was subdivided into three parts, where all six mARG-clusters shared 60 bp directly upstream from the *lnu(A)* gene (light blue block), and cluster D5 shared 140 bp (pink block) directly upstream from this 60-bp block, but not the remaining 740 bp (light green block). No genes were identified on these flanking regions.

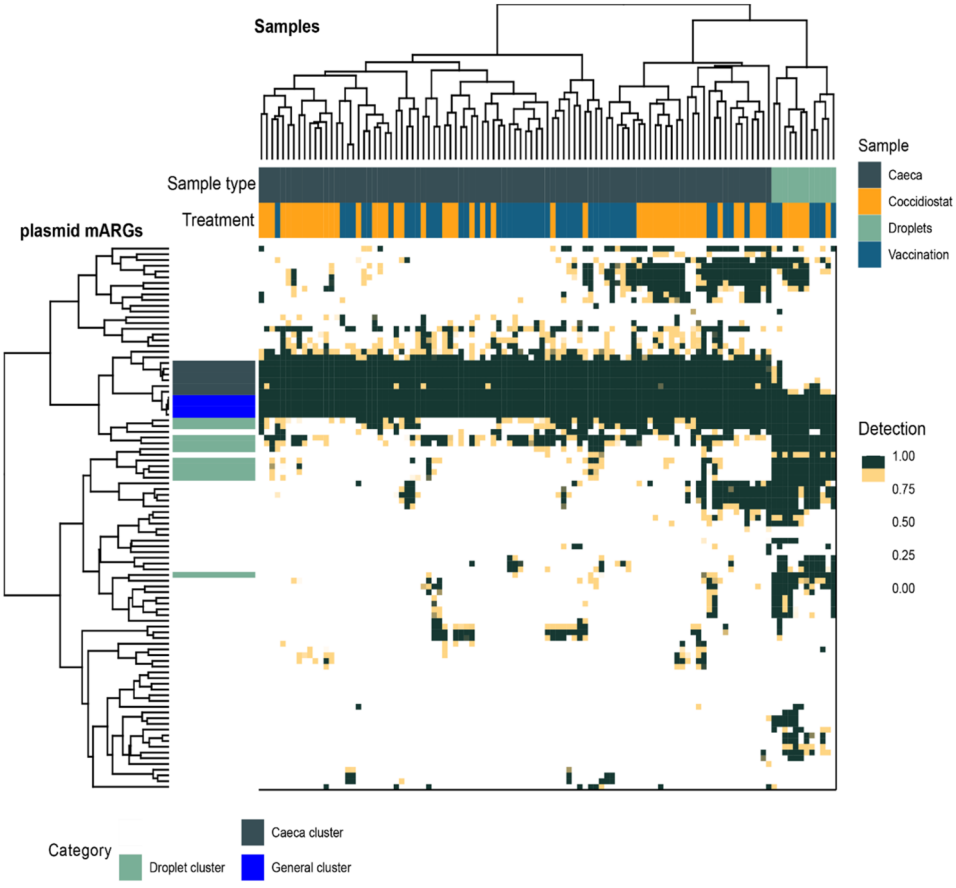

Fig. 3 Detection values per mARG cluster (rows) and sample (columns). Row annotations indicate clusters categorized as dropping-specific, caeca-specific or general when the cluster is present in more than 90% of the samples of a given sample type

Table 2 Antimicrobial resistance genes (ARGs) present on sample-specific plasmid mobile ARG (mARG) clusters, the percentage of samples the clusters are detected in, and the sequence length of the clusters

Origin of cluster	Cluster ID	Detected in % caecal samples	Detected in % droppings samples	Resistance genes	Cluster length (bp)
Caeca	C1	100	50	*tetW*	2020
	C2	100	83.33	*tet(O)*	6447
	C3	100	83.33	*tet(O)*	2726
	C4	100	83.33	*tet(O)*	4053
	C5	100	83.33	*tet(O)*	5159
	C6	94.74	33.33	*erm(G)*	13,667
Faecal dropping	D1	42.11	100	*lnu(G) - ant(3")-Ia*	3667
	D2	42.11	100	*lnu(A) - ant(9)-Ia*	2579
	D3	63.16	100	*lnu(A) - ant(9)-Ia*	3421
	D4	21.06	100	*ant(9)-Ia*	2086
	D5	10.53	100	*lnu(A) - ant(9)-Ia*	2019
	D6	52.63	100	*lnu(A) - ant(9)-Ia*	2497
	D7	29.47	100	*lnu(A)*	3929
	D8	52.63	100	*lnu(A) - ant(9)-Ia - lnu(A)*	7893
	D9	47.37	100	*ant(3")*	2141
	D10	46.32	100	*ant(9)-Ia - lnu(A)*	2840
General	G1	100	100	*lnuC*	6505
	G2	100	100	*tetW*	2293
	G3	100	100	*tet(W)*	2776
	G4	100	100	*tet(W)*	2687

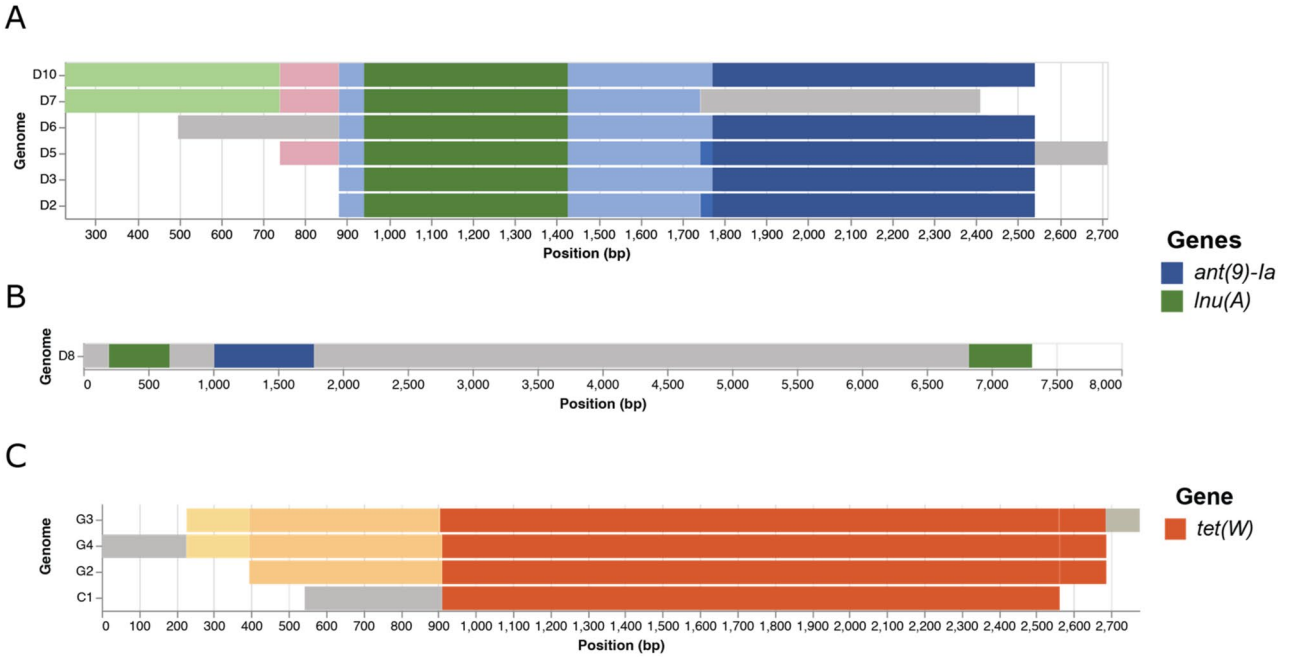

Fig. 4 Aligned flanking regions of selected mobile ARG (mARG) clusters. Panels **A**–**C** show the genomic context of specific ARGs. Homologous regions between clusters are indicated by identical colors (nucleotide sequences), while grey blocks represent regions not homologous to any other cluster. The central ARG is highlighted (e.g., lnu(A), ant(9)-Ia, tet(W)). (**A**) Dropping-specific mARG-clusters containing lnu(A) and ant(9)-Ia genes. (**B**) A dropping-specific mARG-cluster (D8) containing two lnu(A) genes flanking one ant(9)-Ia gene. (**C**) tet(W) mARG-clusters; the top bar indicates the approximate location of the tet(W) gene. Clusters G2, G3, and G4 are generally present across all samples, while mARG-cluster C1 is specific to caecal samples

mARG-clusters D7 and D10 differed in the presence of the *ant(9)-Ia* gene, where D7 lacked the *ant(9)-Ia* entirely. mARG-clusters D2 and D3 were highly identical but differed in a short region (20 bp) directly upstream of the *ant(9)-Ia* gene. This suggests these six mARG-clusters represent up to six distinct plasmids. The composition of the regions flanking the two *lnu(A)* genes and the *ant(9)-Ia* gene in cluster D8 was distinctly different from

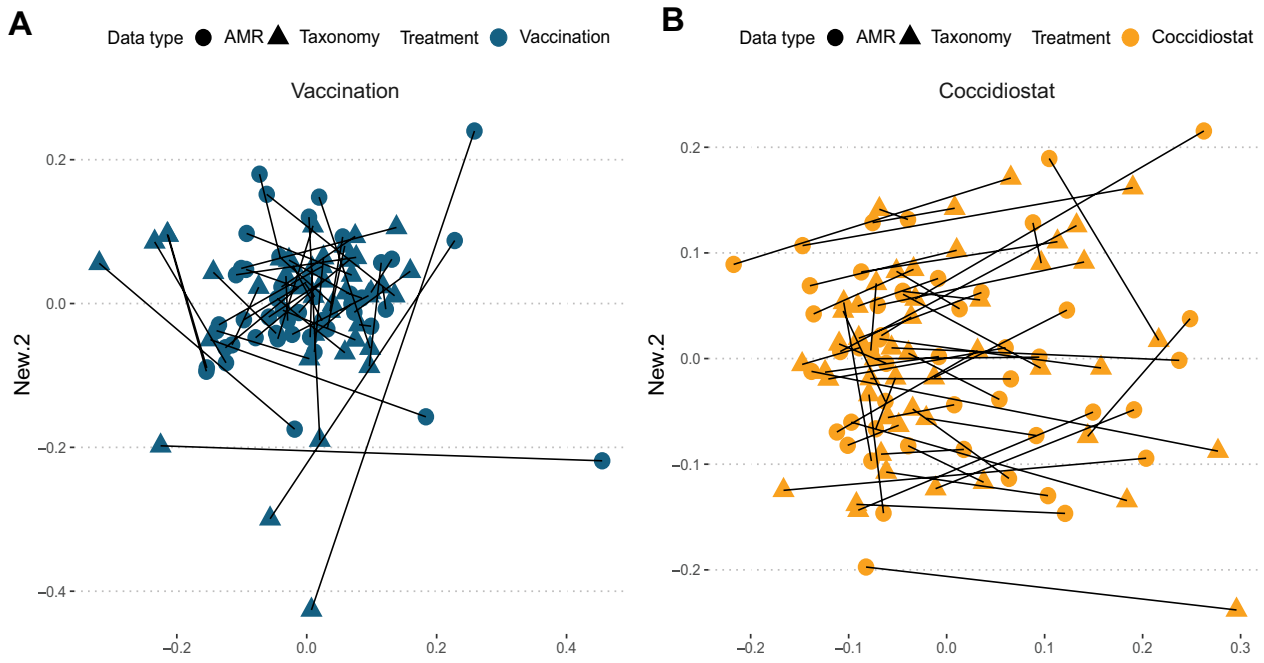

Fig. 5 Procrustes rotation between ordinations of resistome and microbiome compositions in caecal samples for vaccine (**A**) and coccidiostat (**B**) intervention groups. Shapes indicate data type, colors indicate treatment. Connected points are the same sample, their line indicating the error distance. Both groups showed significant correlation (Vaccination: correlation coefficient = 0.690, $p < 0.001$; Coccidiostat: correlation coefficient = 0.601, $p < 0.001$)

the clusters containing only one *lnu(A)* gene. This can therefore be seen as another distinct plasmid fragment in which these genes occur.

To determine the genetic context of the *lnu(A)*/*ant(9)-Ia* mARG-clusters, we used BLASTn against the refseq genomes and nt databases. Notably, only cluster D3, which only contained the region including the *lnu(A)* and *ant(9)-Ia* genes, had a match spanning across the entire sequence (100% query coverage), as displayed in Supplementary Table 6. Of the other mARG-clusters, no match was found that covered both the *lnu(A)* and *ant(9)-Ia* genes, as well as both flanking regions. The matched D3-cluster sequence is from plasmids isolated from *Limosilactobacillus reuteri* in a sample of pig faeces, and isolates of *Enterococcus gallinarium* and *Enterococcus caselliflavus* from surface water samples. The *Enterococcus* plasmids differed from the D3 mARG-cluster as these plasmids contained an *erm(A)* pseudogene and a *mobM* gene between the *lnu(A)* and *ant(9)-Ia* genes, that were absent in all mARG-clusters. None of the flanking regions of the other mARG-clusters matched these plasmids, indicating these mARG-clusters originate from plasmids currently missing in public databases.

Of the *tet(W)* mARG-clusters, three were found in the general category, and one was found in the caeca-specific category. The *tet(W)* cluster that was associated with caecal samples contained a different region upstream of the *tet(W)* gene compared to those present in all samples, noted by a difference in the regions surrounding these genes. The other *tet(W)* mARG-clusters were highly similar in terms of sequence similarity, but varied in length (between 2,293 and 3,556 bp). It is possible that these three clusters belonged to a similar plasmid but were assembled into contigs with different lengths, preventing their co-clustering in this analysis.

The Microbiome shapes the resistome

To detect correlations between the ordinations of the bacterial composition and the resistome compositions, we used a procrustes analysis on the taxonomy and the resistome PCoA ordinations. This revealed significant correlation coefficients of 0.516 ($p < 0.001$) in the caeca and 0.805 ($p < 0.001$) in the faecal droppings samples, between the gene-level resistome composition and genus-level taxonomic composition. We also checked whether the observed correlations were specific to the prevention intervention. We observed for the caeca samples a correlation coefficient of 0.690 ($p < 0.001$) in the vaccinated and 0.601 ($p < 0.001$) in the coccidiostat samples, while for the faecal dropping correlation coefficients of 0.927 ($p < 0.001$) and 0.789 ($p < 0.01$) were observed in the coccidiostat and vaccination samples, respectively.

The rotated ordination of the caecal samples divided by intervention is displayed in Fig. 5; the rotated ordination of the faecal droppings samples is displayed in Figure S8. This correlation suggests that the microbiome shapes the resistome and that this correlation is present in both interventions.

Discussion

In this exploratory study, we compared the effects of two broiler coccidiosis prevention strategies, a coccidiostat and an anticoccidial vaccination, on the composition and interaction of the gut microbiome and resistome, using metagenomics sequencing and targeted resistance gene capture. Additionally, we applied a novel method to characterise putative mobile ARGs and compared their presence in caecal and faecal dropping samples per intervention. This method allowed for the identification of mARGs with the putative potential of spreading inside and outside the farm environment.

We observed no significant difference in within-sample taxonomic diversity (alpha diversity) between interventions but found a significant difference in between-sample taxonomic diversity (beta diversity) in both caecal and faecal dropping samples. Similar intervention-specific effects were noted in previous studies, where the inclusion of vaccination [47] and narasin [48], also in combination with nicarbazin [49], led to differences in bacterial composition in the caecal gut microbiome of broilers.

Comparing the relative abundance of bacterial phyla between interventions in the caecal samples showed a reduction of the ratio of *Bacillota (*formerly *Firmicutes*) to *Bacteroidota* (formerly *Bacteroidetes)* in the coccidiostat samples. Although not reported directly, this was previously observed in other studies on the effect of coccidiostats on the broiler microbiome [47, 48]. This ratio plays a role in mediating the transition from the primary utilisation of *Bacillota*-specific butyrate-producing pathways earlier in life to a balance between *Bacillota* butyrate-producing pathways and *Bacteroidota* butyrate- and propionate-producing pathways [50]. Butyrate is an important product of caecal fermentation associated with poultry gut health and is associated with anti-inflammatory functions [50–52], contributing to gut barrier integrity and immune modulation [53]. The differences in abundance of *Bacillota* and *Bacteroidota* play a role in the efficiency of energy conversion and vary with age and dietary interventions [54, 55]. Furthermore, comparing the abundance of bacterial genera between interventions, we found 32 genera differentially abundant between vaccination and coccidiostat caecal samples. Specifically, we observed a significant reduction in the relative abundance of important butyrate-producing *Bacillota* like *Faecalibacterium*, *Gemmiger*, *Intestinimonas* and *Butyrococcus*, in broilers that received coccidiostats relative to the vaccinated group as well as *Lactobacillus*. *Lactobacillus* can be beneficial to gut health [54] in certain stages of development but has a variety of interactions, positive and negative, with other members of the caecal microbiome [55–57]. These beneficial effects often include competitive exclusion of pathogens and localized immune improvement against *Eimeria* species [58, 59]. Similar findings on the relative abundance of these genera have been reported previously both in vitro [60] and in vivo [61].

Conversely, members of the bacterial genera *Alistipes* and *Bifidobacterium* were found in higher relative abundance in the coccidiostat group compared to the vaccinated group. *Alistipes* is a genus of bacteria producing acetic acid that has been associated with higher nutrient retention variables [62] and is most abundant at a later stage of gut microbial development [63]. Furthermore, *Bifidobacterium*, a genus that has been shown to be a stable part of the core caecal microbiome over time [64, 65], is a genus of beneficial lactic acid producers [66].

These changes in relative abundance suggest that preventive intervention using coccidiostats may lead to a shift towards a more mature microbiome through direct modification of the bacterial community composition, which may impact the metabolic repertoire of the gut microbiome in broilers. Although we did not investigate the influences of this shift on general health, a more mature-associated microbiome can play a positive role in gut health and a stronger immune system [67, 68]. Although differential abundance analysis using ANCOM-BC2 on the faecal dropping samples did not reveal statistical significance due to the low sample number [32], we observed a higher relative abundance of members of the genera *Lactobacillus*, *Faecalibacterium* and *Limosilactobacillus* in the vaccinated samples and a higher relative abundance of *Escherichia*, *Streptococcus* and *Ligilactobacillus* in the coccidiostat-treated samples. This is in line with our observations in the caecal samples.

In accordance with previous studies [69–71], we observed a notable difference between the taxonomic composition of caecal content and faecal droppings. This shift in microbiome composition is most likely due to fact that faecal droppings are mostly of ileal origin and that differences in specific conditions, especially environmental oxygen exposition, which may favour the overgrowth of strict anaerobic bacteria by aerobic or facultatively anaerobic bacteria, were present at sample collection. Although faecal droppings are a less representative source for analyzing the composition of the caecal microbiome of broilers, they are produced in higher abundance compared to caecal droppings and thus may be more relevant with respect to environmental transmission, including potential animal-to-human transmission, of bacterial clones and their (mobile) genes. Direct statistical comparison between sample types in microbiome composition, as well as resistome and mobilome abundances, is not possible due to the difference in sampling and sequencing strategies, where the pooling of the faecal droppings has an effect on the relative sequence composition of the sample.

In addition to the taxonomic composition, we investigated the resistome composition to evaluate the influence anticoccidial intervention had on the broiler resistome. Our analysis also revealed distinct patterns in ARG class distribution, with rifampicin resistance exclusively in faecal droppings, nitroimidazole only in caeca, and quinolone resistance genes uniquely associated with coccidiostat samples. The latter observation is particularly notable given that quinolones are critically important antimicrobials in both animal and human medicine, raising significant public health implications [72]. Some ARGs were found to be differentially abundant between interventions in both sample types, like *vanHAX*, *tet(L)* and the *cat* gene on the pC194 plasmid. In some cases, the differential abundance between interventions seemed to be compensated by a higher abundance of other related genes. For example, *tet(M)* and *tet(L)* were more abundantly in the vaccinated group compared to the coccidiostat group in the caeca samples, whereas *tet(A)* was more abundant in the coccidiostat caecal samples relative to the vaccination group. The observation that *tet(M)* and *tet(L)* were found more abundant in the vaccination group than in the coccidiostat group could be attributed to the fact that genera like *Lactobacillus* and *Limosilactobacillus*, associated with specific resistance genes such as *cat*, *cfr*, *tet(M)* and *tet(L)* [73], were also found to be more abundant in the vaccinated group relative to the coccidiostat group. This was in line with the procrustes analysis which indicated correlations between the taxonomic composition and the resistome.

We found 21 different ARGs to be over- or underrepresented in one of the interventions in either the caecal or the faecal dropping samples. The *vanHAX* operon was one of the genes more abundant in the vaccination group than in the coccidiostat group [74, 75]. This operon consists of three genes (*vanH*, *vanA* and *vanX*) that together with the two regulatory genes *vanR* and *vanS* encode for vancomycin resistance. Although the *vanA* gene was previously reported to be associated with the narasin resistance *narAB* operon [76], we found that the *vanHAX* operon was less abundant in the coccidiostat samples, where *narA* and *narB* were more abundant, compared to the vaccinated samples. Additionally, we were unable to detect the presence of the *vanHAX* operon in the ResCap data of the coccidiostat samples with a threshold of one fragment per kilobase of gene. As all samples originated from the same farm, it is possible that the plasmids conferring co-resistance between narasin and vancomycin are not circulating on this farm, but the fact that *vanHAX* operon was found to be more abundant in the vaccinated samples requires further investigation. In the coccidiostat faecal dropping samples, the gene most differentially abundant was the *qnrs7* gene, which causes resistance to quinolone. Furthermore, *DfrA1* was also found in higher abundance in coccidiostat-treated faecal droppings samples. This trimethoprim resistance gene has been previously shown to be co-selected on plasmids harboring *qnrS* genes [77, 78]. However, neither *qnrS* nor *dfrA1* were found in our analyses on the mobile fraction of ARGs, possibly due to the low abundance of these genes, denoted by the low regularised log values in Fig. 2, preventing the correct assembly of these plasmids. Our ResCap data showed that no *qnrS7* genes were found present in any of the vaccinated samples, regardless of sample type.

In addition to metagenomics sequencing, applying ResCap allowed the detection of additional resistance genes. However, given the nature of the technique, which involves probe hybridization and gene amplification via polymerase chain reaction (PCR), differential abundance could not be inferred. With ResCap, we were able to detect ARGs that were widely distributed, yet not detected in the metagenomic data of the tested sample population, especially in the faecal droppings, most likely due to their low abundance in the gut. This might be because the technique, through probe hybridization and DNA amplification, overcomes limited detection of very low abundance genes. Moreover, in the faecal droppings the better detection could be explained by the fact that the droppings samples have been pooled, leading to a reduction of variance due to the relative overrepresentation of common sequences compared to the relative underrepresentation of rare sequences. On the other hand, the set of ARGs that was not identified by ResCap but was detected in the metagenomics data were only present in a few samples. For most of these genes (26/33), this is a result of discrepancies in the version of the ResFinder database used for the design of the capturing probes of the ResCap technique in comparison with the version used for the analysis of the metagenomics data.

Similar to our observation for the taxonomic composition, ARG composition (beta diversity) was found significantly different between the different anticoccidial strategies in the caecal samples. Previous work has shown that different bacterial communities harbour different resistome profiles [36, 79]. In the same line, we observed a significant correlation between taxonomic and resistome compositions that shows that the resistome is influenced by the bacterial community in both sample types.

Although no clear distinction was found between interventions in the overall presence of mARG-clusters, we did observe specific mARG-clusters that were more prevalent in the coccidiostat group. Specifically, four mARG-clusters in the faecal droppings samples, containing a tetracycline resistance gene [*tet(A)*], were more prevalent in the coccidiostat group than the vaccinated one, which aligned with the higher abundance of *tet(A)*

in coccidiostat faecal dropping samples. Notably, in one mARG-cluster, we found *tet(A)* in association with the ampicillin resistance-encoding gene $bla_{\text{TEM-1B}}$. This specific co-occurrence, more pronounced in coccidiostat-treated groups, could suggest a co-selection pressure exerted by ionophores that favors the mobile carriage of these ARGs. These findings are in line with a previous study investigating the co-occurrence of ionophore resistance in enterococci isolated from poultry with other clinically relevant types of antimicrobial resistance, where a significant correlation was observed between coccidiostat and both tetracycline and ampicillin resistance [10]. Overall, this suggests that a *tet(A)*, also in combination with bla_{TEM-1B}, may be possibly located on a plasmid and thus potentially transferable to the farmhouse environment, more frequently in the coccidiostat samples than in the vaccination samples.

Of the four mARG-clusters found in all samples of both sample types, three of them contained a *tet(W)* gene. This gene has been found prevalent in farmhouse dust on poultry farms but is also highly abundant in the human gut [80]. It is therefore not surprising to find this gene present in both sample types. However, this gene can be present in multiple different genetic contexts, not restricted to associations with plasmids or transposons [81, 82]. Depending on the bacterial species, the spreading of these mobile elements can therefore still contribute to between-environment dissemination, as the genes seem to persist in the faecal droppings on the farmhouse floor.

Another mARG cluster present in both sample types contained an *lnu(C)* gene. This gene, conferring lincosamide resistance, is transferable by a transposon, and can be found in both chromosomal and plasmid contexts [83]. This gene has been found to be associated with chicken farming but is also commonly present in the human gut microbiome [84, 85], although high prevalence is more commonly reported in studies that include humans in close contact with livestock animals [83–86].

In six of the mARG-clusters that were only associated with the faecal dropping samples, an *ant(9)-Ia* and an *lnu(A)* gene were found in tandem, in different genetic configurations based on their flanking regions. This combination of genes is therefore likely to be present in multiple plasmid or transposon contexts, and since it's present in the environmental faecal samples, it can presumably spread around the farmhouse and other environments (e.g., litter is also often used as biomass energy). The striking variability in the flanking regions of these *lnu(A)*-containing mARG-clusters, encompassing shared upstream blocks and distinct genetic contexts, underscores their significant genetic plasticity and active mobilization. This poses a considerable risk by providing abundant opportunities for recombination, e.g., homologous recombination via shared sequences or site-specific integration, which can rapidly integrate mARGs into novel genetic environments, accelerating their dissemination and intensifying the spread of antibiotic resistance. Monitoring these mARGs in real-time on farms could involve targeted qPCR assays or periodic metagenomic surveillance of environmental samples (e.g., litter, dust, water) to track their prevalence and mobility.

lnu(A), conferring lincosamide resistance, is often found on plasmids [87, 88] of *Staphylococcus* species. However, in these samples, a conclusive species assignment of these plasmids could not be given. This can be explained by the fragmentation of the assembly and consequent short flanking regions, and partially because no match could be found with known plasmids that contained these genes in the same genetic configuration. The core region containing only the *ant(9)-Ia* and *lnu(A)* genes did match plasmids sequenced in pig faeces and surface water, in multiple genera, further suggesting that these are plasmid-borne resistance genes. Whether these plasmids have the capacity to spread to the environment and to human hosts is currently unknown.

With this exploratory study, we provide valuable insights that should be interpreted with caution due to several limiting factors. The intrinsic variability between consecutive flocks might be responsible for part of the observed variation. Crucially, the study design, involving two consecutive flocks from a single farm, confounds the anticoccidial strategy with farm-specific factors, thus preventing definitive causal conclusions and limiting the generalizability of our findings. Previous research has shown that even control groups in separate experiments can exhibit distinct microbiome compositions [89]. This is because, in the first weeks of life, chicks are highly sensitive to bacterial colonization [90], which can introduce variability in microbiome and resistome composition across flocks [91]. Throughout the broiler's life, this early colonization may have a lasting impact on the development of its microbiome and, possibly, how it reacts to interventions. Therefore, to separate intervention-specific effects from natural variability, future research may include repeated sampling across several farms and flocks. Furthermore, as our analyses relied on relative abundance metrics observed shifts may not directly reflect changes in absolute quantities of microbial taxa or ARGs, potentially obscuring true biological changes and impacting community relationship interpretations. To address this, future studies could employ statistical methods specifically designed for compositional data analysis to better infer underlying biological shifts [92].

Conclusions

Our findings demonstrate the impact of anticoccidial strategies on the chicken gut microbiome and resistome with potential consequences for the dissemination of ARGs. While the microbiome clearly shapes the resistome, anticoccidial interventions can selectively modify both, indirectly influencing the ARG landscape. The identification of mARG-clusters in faecal droppings underscores the potential for ARG dissemination into the environment, with some clusters— including those harboring *tet(A)* and bla_{TEM-1B}, more prevalent in the coccidiostat group, suggesting possible co-selection mechanisms that warrant further investigation. Finally, we can conclude that within the scope and limitations of this exploratory study, no specific intervention resulted in a higher risk of AMR selection. Further investigation, ideally on multiple flocks to account for flock-to-flock variability, along with functional validation to confirm their transferability, would help to better understand the impact of coccidiosis prevention on the ARM gene pool.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Not applicable.

Author contributions
ACS, RJW, FLP and HE contributed to the conception and contributed to the design of the work. MB contributed towards samples collection, processing and sequencing. MB and JJK provided data analysis and interpretation. MB and JJK drafted the manuscript. All the authors read, edited and approved the final manuscript.

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Data availability
Sequencing data have been made available on the European Nucleotide Archive under project PRJEB82955. An overview of software versions used in this study can be found at [https://gitlab.com/mmb-umcu/broiler_resistome](https:/gitlab.com/mmb-umcu/broiler_resistome) .

Declarations

Ethical approval and consent to participate
The animal experiments were performed in accordance with permissions of the Dutch Ethical Committee for the sacrifice of broilers on a production farm by cervical dislocation on a small scale.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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