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Extended period of selection for antimicrobial resistance due to recirculation of persistent antimicrobials in broilers

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Objectives: Antimicrobials can select for antimicrobial-resistant bacteria. After treatment the active compound is excreted through urine and faeces. As some antimicrobials are chemically stable, recirculation of subinhibitory concentrations of antimicrobials may occur due to coprophagic behaviour of animals such as chickens.

Methods: The persistence of three antimicrobials over time and their potential effects on antimicrobial resistance were determined in four groups of broilers. Groups were left untreated (control) or were treated with amoxicillin (unstable), doxycycline or enrofloxacin (stable). Antimicrobials were extracted from the faecal samples and were measured by LC-MS/MS. We determined the resistome genotypically using shotgun metagenomics and phenotypically by using *Escherichia coli* as indicator microorganism.

Results: Up to 37 days after treatment, doxycycline and enrofloxacin had concentrations in faeces equal to or higher than the minimal selective concentration (MSC), in contrast to the amoxicillin treatment. The amoxicillin treatment showed a significant difference ($P \leq 0.01$ and $P \leq 0.0001$) in the genotypic resistance only directly after treatment. On the other hand, the doxycycline treatment showed approximately 52% increase in phenotypic resistance and a significant difference ($P \leq 0.05$ and $P \leq 0.0001$) in genotypic resistance throughout the trial. Furthermore, enrofloxacin treatment resulted in a complete non-WT *E. coli* population but the quantity of resistance genes was similar to the control group, likely because resistance is mediated by point mutations.

Conclusions: Based on our findings, we suggest that persistence of antimicrobials should be taken into consideration in the assessment of priority classification of antimicrobials in livestock.

Introduction

Antimicrobial compounds are crucial and lifesaving medicines, which can be applied to treat infections with a bacterial pathogen and preventatively for surgery or organ transplantations.[1–3] Unfortunately antimicrobial resistance (AMR) has become a growing problem during recent decades.[4] The rising development of AMR has been considered as one of the most serious health threats for humans and animals by organizations such as the WHO, the Food and Agriculture Organization of the UN (FAO) and the World Organisation for Animal Health (WOAH).[3,5,6] The excessive use of antimicrobials in livestock is contributing to the emergence of resistant bacteria.[7] Measures to reduce antimicrobial usage in livestock are important to reduce the exposure to antimicrobial concentrations that select for resistant bacteria.

In terms of reducing worldwide antimicrobial usage, antimicrobial stewardship programmes have been established.[8,9] These programmes consist of guidelines to reduce the use of antimicrobials and to stimulate prudent usage of antimicrobials in livestock.[10–12] For example, the EMA has classified antimicrobial compounds into four different categories; A (avoid), B (restrict), C (caution) and D (prudence).[13] This categorization of antimicrobial compounds has been established to reduce potential consequences for public health as a result of increased AMR.

Based on the classification of the EMA, antimicrobials are selected and applied by veterinarians to limit the selection of

AMR. However, in this categorization, only direct selection during treatment is considered. Studies have shown that there is an extensive difference in half-life properties between antimicrobial compounds, ranging from hours to over a year.[14–16] This implies that, for specific antimicrobials, selection for resistant bacteria can be maintained long after the application and withdrawal time.[16,17] Especially in poultry, the persistence of antimicrobial compounds is of concern due to coprophagic behaviour, which induces recirculation of the antimicrobial compounds, thereby re-exposing the gut microbiome to residual concentrations that may be above the minimal selective concentration (MSC) for a longer period, resulting in prolonged selective pressure.[17–20] Since persistence is not included in the assessment of the classification of antimicrobials it could be a missed opportunity for reducing AMR. To elaborate, to our knowledge, no studies have been conducted that show a relationship between persistence of antimicrobial residues and resistance levels.

The current study measured the temporal antimicrobial residue concentration in faecal droppings and caecal material after treatment of broilers. Alongside this, we investigated the prevalence of AMR in *Escherichia coli* isolates and the resistance genes in resistome data after cessation of the antimicrobial treatment. An animal trial was conducted, in which groups of broilers were treated with amoxicillin (unstable), and doxycycline and enrofloxacin (stable). Antimicrobials were extracted from the faecal samples and analysed by LC-MS/MS and were compared with the MSCs determined by A. F. Swinkels, E. A. J. Fischer, L. Korving, N. E. Kusters, J. A. Wagenaar, A. L. Zomer (unpublished data). We performed phenotypic susceptibility testing by plating *E. coli* isolates. Additionally, we quantified the read depth of resistance genes present in the resistome to investigate resistance genes besides the indicator organism *E. coli*. Lastly, we studied whether the antimicrobial residuals had any effect on the composition of the microbiome.

Materials and methods

Housing of the broilers, experimental set-up and sample collection

A total of 158 broilers (commercial Ross 308) were included in this trial and were transported from the hatchery to the animal facility (Utrecht University, Utrecht, The Netherlands) on the day of hatching (Day 0 of their life). The broilers were weighed, tagged and housed in one pen (6 m^2) to equilibrate their microbiome. After 4 days, caeca were collected after euthanizing 12 broilers. Subsequently, the remaining broilers were randomly divided into different treatments groups (Day 0 of the experiment) and treatment started. The groups were treated with amoxicillin, doxycycline or enrofloxacin and an untreated control group. Each group was divided into three subgroups containing 12 broilers. The groups stayed in separate stables with hygiene rules to prevent carry-over of antimicrobials and bacteria. The antimicrobial treatment lasted for 4 days and was administered via the drinking water. Doses that were used were 25 mg per kg of bodyweight per day for doxycycline, 10 mg per kg of bodyweight per day for enrofloxacin and 20 mg per kg of bodyweight per day for amoxicillin.[21–23] Afterwards, faecal droppings were collected individually from the broilers placed in carton boxes with paper at the bottom and were homogenized to obtain pooled samples. A fraction was stored at −80°C to quantify the presence of antimicrobials. This was repeated at Days 6, 12, 19 and 26 after the start of the treatment. On Day 37, after treatment, broilers were euthanized, and caecal material was collected. The samples were transported to the lab and further processed.

Ethics of experimentation

Broilers were observed daily for the presence of clinical signs, abnormal behaviour and mortality. The study protocol was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee of Utrecht University (Utrecht, The Netherlands) under registration number AVD10800202114909. All procedures were done in full compliance with all legislation. A power calculation was conducted to estimate the total broilers needed for the experiment, based on a simulation performed in R (Supplementary data, available in Zenodo repository; https://zenodo.org/records/11103906).

The size of the pens of the subgroups was set to 2 m^2, according to the legal regulations in Appendix III of 2010/63/EU[24] to not exceed the kg/m^2. Loss of broilers in the first week of the experiment due to health issues was expected, resulting in 12 broilers per subgroup. Solely female broilers were selected to reduce weight increase.[25]

Phenotypic susceptibility testing

From every subgroup and timepoint, a swab was used to inoculate the pooled samples on three MacConkey plates. Next, 24 single colonies were picked by a pipette tip after overnight culture at 37°C and transferred to a single well in a 96-well plate with 100 µL of LB medium per well. The 72 colonies per treatment and timepoint in one 96-well plate were transferred to square MacConkey plates with epidemiological cut-off value (ECOFF) concentrations of amoxicillin (8 mg/L), doxycycline (4 mg/L), enrofloxacin (0.125 mg/L) and a control plate without antimicrobials via a stamp. Next, non-WT (NWT) colonies were scored after overnight incubation at 37°C. An *E. coli* colony was scored as NWT if it were able to grow on the selection plate with the respective antimicrobial. We calculated *E. coli* resistance by comparing growth on control and selection plates.

Shotgun metagenomics

DNA was extracted according to the 'Ecology from Farm to Fork Of microbial drug Resistance and Transmission' project (EFFORT) protocol; DNA concentrations were measured with a Qubit.[26,27] Illumina sequencing was performed using an Illumina NovaSeq 6000 (USEQ, Utrecht sequencing facility) with a maximum read length of 2×150 bp. Libraries were prepared with the Illumina Nextera XT DNA Library Preparation Kit according to the manufacturer's protocol.[28] Each read was trimmed with Trim Galore (v0.6.4_dev) and the quality was assessed with FastQC (v0.11.4). The reads were analysed for taxonomic classification by Kraken2 and the abundance of the DNA sequences was computed with Bracken.[29,30] The output was summarized into a biom file using kraken2-biom and was analysed with the R programme packages phyloseq (v1.36.0), microViz (v0.9.1) and microbiome (v1.14.0), by which the species composition, and alpha and beta diversity were estimated from a rarefied phyloseq object.[31,32] Finally the resistome was investigated by using KMA (v1.4.2) utilizing the ResFinder database with a minimum of 80% gene coverage.[33,34] The reads were first normalized for gene length and displayed as sequence depth per Gb of sequencing data (sequence data for this article can be found in the SRA under accession PRJEB73721).

Antimicrobial analysis of the faecal samples

The faeces samples were stored at −80°C until extraction of the antimicrobial compounds. The samples were analysed according to the procedure described by Berendsen *et al.*[14] In short, from the samples, approximately 1 g was weighed into a 50 mL polypropylene (PP) centrifuge tube and internal standard solution was added. The antimicrobial compounds were extracted by 4 mL of McIlvain-EDTA buffer and 1 mL of acetonitrile. Afterwards, 2 mL of lead acetate solution was added to remove excessive proteins. After centrifugation, the extract was transferred to a clean test tube and diluted by 13 mL of 0.2 M EDTA.

A Phenomenex (Torrance, CA, USA) Strata-X RP 200 mg/6 mL reversed phase solid phase extraction (SPE) cartridge was conditioned with 5 mL of MeOH and 5 mL of water. The complete extract was applied onto the SPE cartridge and washed with 5 mL of water before being vacuum-dried for 5 min. The antimicrobial compounds were eluted by adding 5 mL of methanol to the cartridges. The eluate was then evaporated at 40°C under nitrogen. The residue was redissolved in 200 µL of methanol and 300 µL of water was added before transferring the extract into an LC-MS/MS sample vial.

The LC system consisted of a Shimadzu UFLC XR (Milford, MA, USA) model Acquity with a Phenomenex Kinetex C18 analytical column of 1.7 µm C18 100 Å, 100×2.1 mm, placed in a column oven at 40°C. The gradient profile with a flow rate of 0.3 mL/min is shown in Table 1. The injection volume was 5 µL. Detection was carried out by LC–MS/MS using a Sciex (Framingham, MA, USA) Q-Trap 6500 mass spectrometer in the positive electrospray ionization (ESI) mode. The antimicrobials were fragmented using collision-induced dissociation (N_2) and the scheduled Selected Reaction Monitoring (SRM) transitions (20 s window) as described by Berendsen *et al.*[35] Data were processed using MultiQuant software v2.1.1 (Sciex).

Statistical analysis

We used R (v4.1.0) and the package lme4 (v1.1-29) for statistical calculations and logistic mixed-effects and linear mixed-effects models. Full models included 'treatment' and 'time' and the interaction between treatment and time as fixed effects and 'pen' as random effect. A logistic regression model was fitted with an interaction term for time after treatment and treatment. This was necessary due to inflated standard errors caused by counts of 0 (WT) or 24 (all NWT). Models without time and/or treatment were compared with the full model. The best model was selected based on the Akaike information criterion (AIC). For the resistome data, we performed a *post hoc* Tukey test to determine whether the treatment groups at the different timepoints were significantly different from the control group. The microbiome diversity was measured with the Shannon index for alpha diversity and Bray–Curtis distance for beta diversity. All files are in the Zenodo repository; https://zenodo.org/records/11103906.

Table 1. Gradient profile used in the LC–MS/MS method

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (mL/min)
0.0	99	1	0.3
0.5	99	1	0.3
2.5	75	25	0.3
5.4	30	70	0.3
5.5	0	100	0.3
6.5	0	100	0.3
6.6	100	0	0.3
7.5	100	0	0.3

Results

Antimicrobial residues extracted from the faecal samples before, during and after treatment

The concentrations of the antimicrobial residues extracted from the faecal samples are shown in Figure 1. At timepoint 0 and in the control group we did not measure any antimicrobial compounds. For the unstable antimicrobial amoxicillin we did not find concentrations that reached the MSC. Only at 6 days after the start of the treatment did we measure amoxicillin at 0.05 mg/kg; however, this was below the MSC. On the contrary, the stable antimicrobials doxycycline and enrofloxacin showed relatively high concentrations after treatment. For doxycycline the concentrations were above or within the range of the MSC up to 26 days. Enrofloxacin concentrations were far above the MSC range after treatment and were still in the MSC range at the time of slaughter. These results suggest that doxycycline and enrofloxacin remain effective beyond application.

Quantifying phenotypic resistance of E. coli isolates over time

The *E. coli* isolates that were isolated over time in the different treatment groups are shown in Figure 2. We observed that the *E. coli* isolates from the amoxicillin treatment started with a high proportion of NWT *E. coli* followed by a declining trend comparable to the control group. For the doxycycline treatment we observed a steep increase directly after treatment and a slight decline at timepoint 12 days; however, the majority of the *E. coli* isolates remained NWT to doxycycline up until the

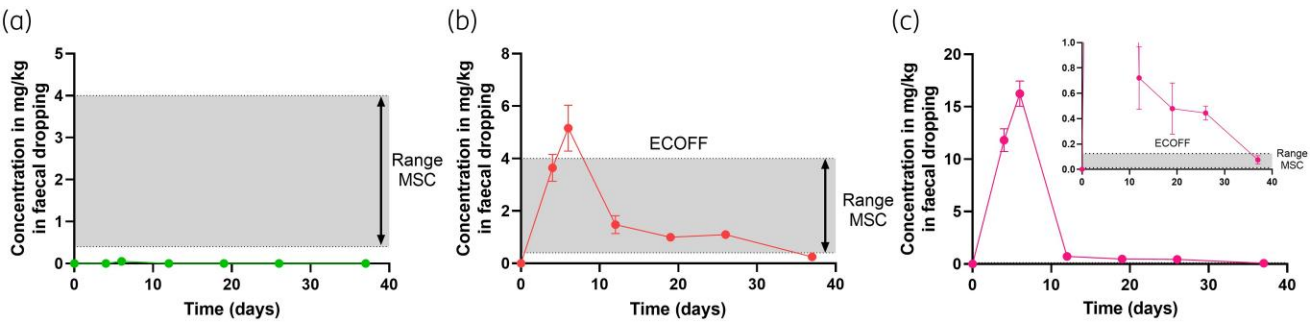

Figure 1. Antimicrobial extraction in faecal droppings of broilers treated with different antimicrobials. (a) amoxicillin, (b) doxycycline and (c) enrofloxacin, which also contains a close-up view with lower concentrations on the *y*-axis to make it more visible. In the graphs, the MSCs are also displayed, determined by A. F. Swinkels, E. A. J. Fischer, L. Korving, N. E. Kusters, J. A. Wagenaar, A. L. Zomer (unpublished data). The MSC was determined by concentrations with a 10-fold difference, therefore the established MSC is displayed as a range between the lowest and highest possible concentration in the figures. The ECOFFs are distinguishing bacteria from WT and NWT. The datapoints are presented as the mean and SEM. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

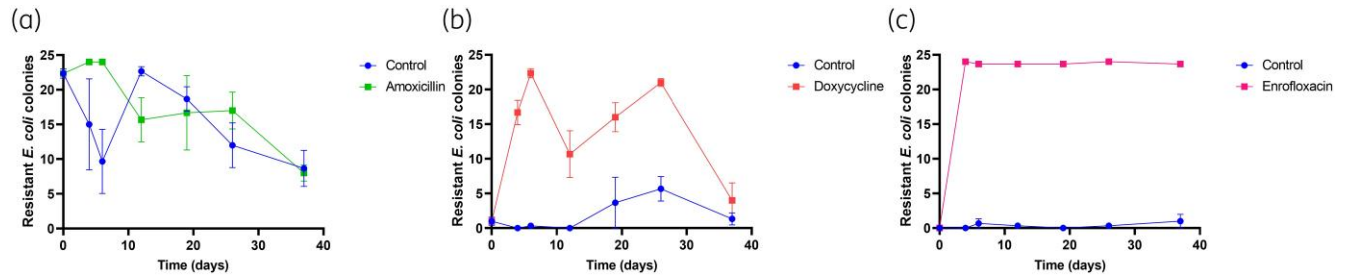

Figure 2. Phenotypic resistance of NWT *E. coli* colonies. The graphs show the resistance of *E. coli* to the antimicrobial with which a group of broilers is treated compared with the control group. (a) Amoxicillin treatment compared with the control group, (b) doxycycline compared with the control group and (c) enrofloxacin compared with the control group. The datapoints are presented as the mean and SEM. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

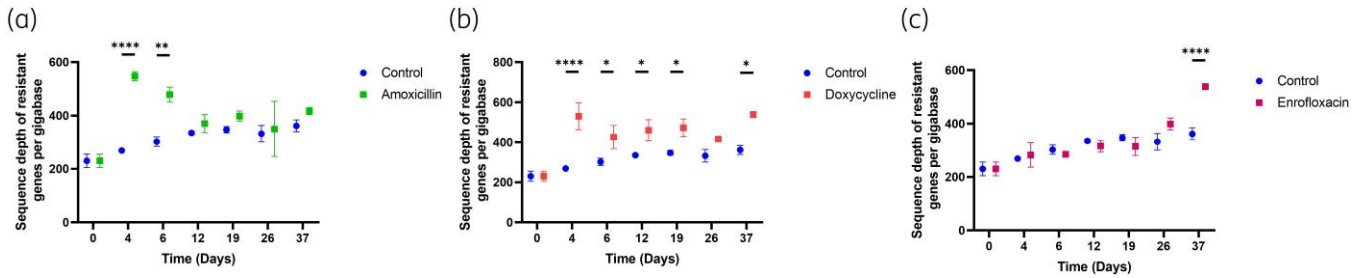

Figure 3. Number of resistance genes in the resistome in sequence depth per Gb over time. (a) The amoxicillin treatment is shown compared with the control group, (b) the doxycycline treatment compared with the control group and (c) the enrofloxacin treatment compared with the control group. The datapoints are presented as the mean and SEM. *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

slaughter age, while in the control group the number of NWT *E. coli* remained limited. For the enrofloxacin treatment we found, almost exclusively, enrofloxacin NWT *E. coli* isolates, which is in strong contrast to the control group where we observed only a few *E. coli* isolates that harboured resistance towards enrofloxacin. The data were best explained (lowest AIC) with a model containing both time after treatment and treatment.

Quantifying resistance genes in faecal droppings

We determined the resistome to quantify resistance genes present in the different treatment groups (Figure 3).

Regarding amoxicillin treatment, we observed more resistance genes immediately after the end of treatment (Day 4) and at Day 6 than in the control group (amoxicillin—control Day 4 $P < 0.0001$, Day 6 $P = 0.0023$). However, by Day 12 after start of treatment, the quantity of resistance genes in the treatment group approached levels similar to those in the control group. Doxycycline treatment led to a similar trend, with increased resistance genes at Day 4 and Day 6 compared with the control group (doxycycline—control Day 4 $P < 0.0001$, Day 6 $P = 0.0161$). However, the difference in resistance genes between the doxycycline-treated group and the control group persisted even at Day 12 ($P = 0.0156$) and Day 19 ($P = 0.0149$). Notably, the first decline in resistance genes within the doxycycline group, approaching control group levels, was observed 26 days after treatment initiation. As for enrofloxacin treatment, its impact on resistance genes was closer to that of the control group, especially up to Day 19. On Day 26, a minor increase in resistance genes was observed but it was not a significant difference. It was only after slaughter that a significant difference was observed in the caecal material between the enrofloxacin-treated group and the control group (enrofloxacin—control Day 37 $P < 0.0001$).

Microbial composition determination after treatment with antimicrobials

We studied the composition of the microbiome to determine if exposure to the residual antimicrobials after treatment had an effect on the microbial composition (Figure 4). The most noticeable change was the *E. coli* abundance in the microbiota of the treated groups. The enrofloxacin treatment resulted in a strong reduction of *E. coli* to almost 0% at Day 4; afterwards it returned slowly in the microbiome. Furthermore, the same was observed for the doxycycline treatment as it reduced *E. coli* to 10% at Day 4 and returned to levels comparable to the control group. On the contrary, the amoxicillin-treated group favoured *E. coli* at Day 4 and its abundance declined over time. Notably, at Day 12, the control group had almost 90% *E. coli* abundance.

In Figure 5 we determined the alpha diversity of the treated groups at the different timepoints. For the amoxicillin treatment we found a lower diversity compared with the control group directly after terminating the treatment ($P = 0.046$). The group treated with enrofloxacin showed a significant difference in Shannon

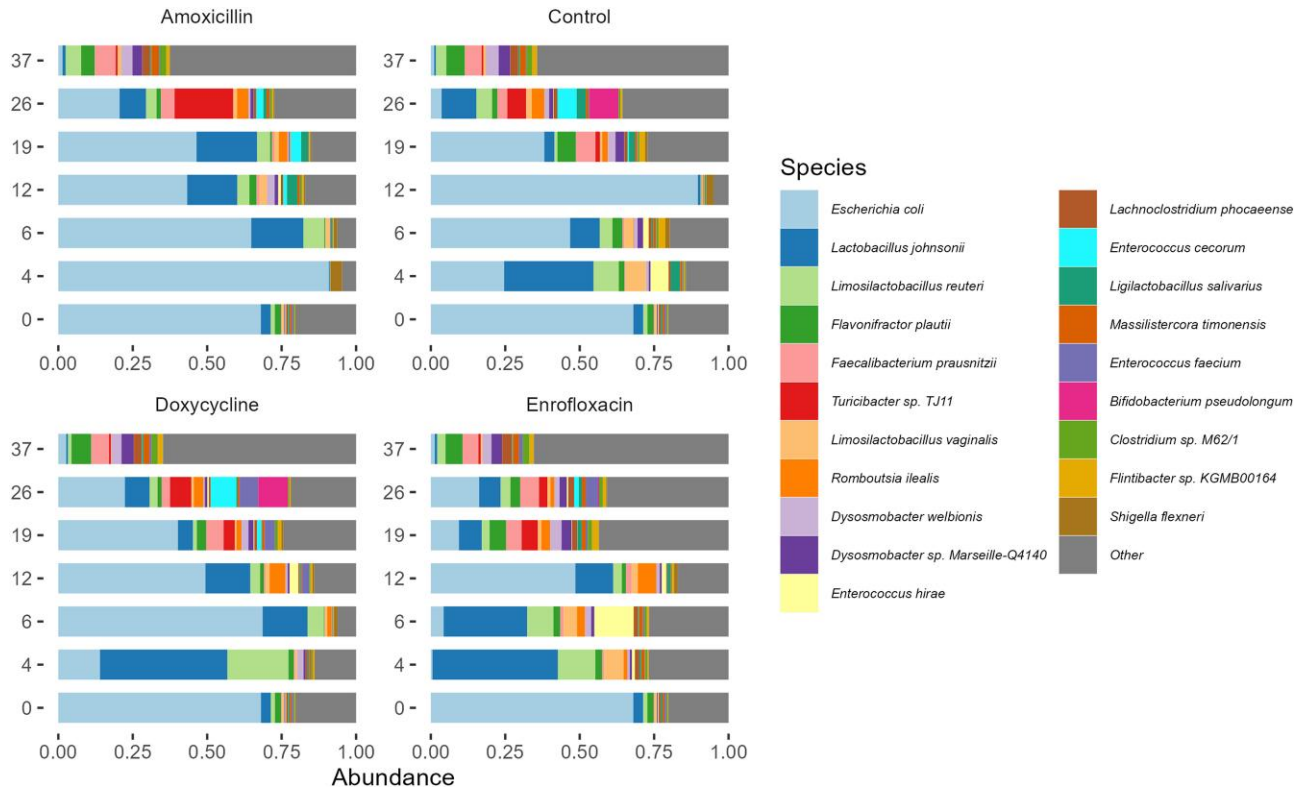

Figure 4. The relative abundance of the samples taken at the different timepoints after treatment. The 20 most abundant taxa are shown in the legend.

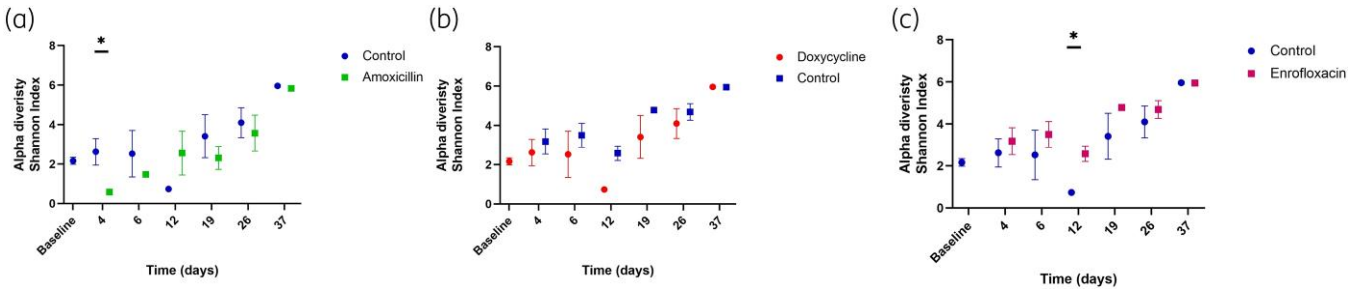

Figure 5. Alpha diversity of the samples at the different timepoints after start of the application. The alpha diversity is measured using the Shannon index. (a) Amoxicillin treatment, (b) doxycycline treatment and (c) enrofloxacin treatment. The datapoints are presented as the mean and SEM. *$P \leq 0.05$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

diversity with the control only at 12 days after start of the treatment ($P = 0.045$). In contrast, the group treated with doxycycline had an equal alpha diversity to the control. We also investigated beta diversity and we calculated the distance from the baseline sample to the samples taken at other timepoints, as shown in Figure 6. The group treated with amoxicillin differed from the control group at 4 days after treatment ($P = 0.015$). Similarly the enrofloxacin treatment group shows a difference 6 days ($P = 0.0062$) from beginning the application to the control group. For the group treated with doxycycline we did not find a difference with the control group.

Discussion

Our findings show that stable antimicrobial compounds like doxycycline and enrofloxacin remain in the faeces of broilers for the length of a broiler production round (41 days) at or above MSC level, resulting in prolonged selection pressure for AMR bacteria. Moreover, these findings are confirming our hypothesis that both doxycycline and enrofloxacin led to higher levels of NWT than the control group, while amoxicillin did not. These results reveal the importance of considering antimicrobial stability when antimicrobials are priority-classified.

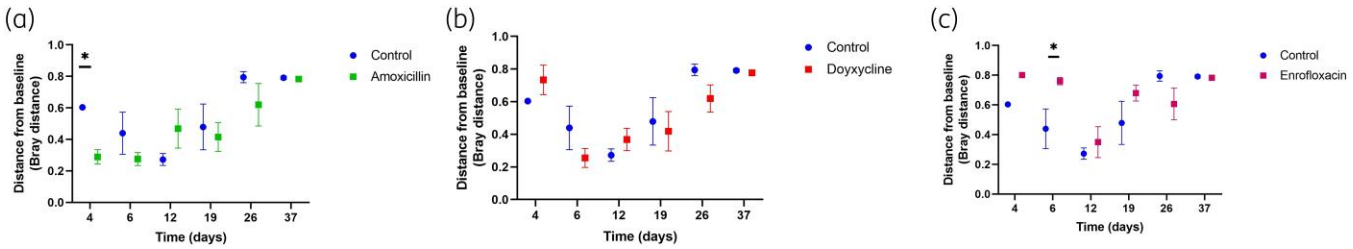

Figure 6. Beta diversity distance of the samples at the different timepoints after start of the treatment; higher distance equals a more different microbiome. The beta diversity is measured with the Curtis–Bray distance and is displayed as distance from the baseline. (a) Amoxicillin treatment, (b) doxycycline treatment and (c) enrofloxacin treatment. The datapoints are presented as the mean and SEM. *$P \leq 0.05$, **$P \leq 0.001$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Worldwide antimicrobial stewardship programmes are implemented to create awareness for judiciously selecting antimicrobial compounds, for example in livestock. The results of this research showed that stable antimicrobials, such as doxycycline and enrofloxacin, can retain selective pressure long after cessation of the treatment: doxycycline from 1.5 to 6 mg/L and enrofloxacin from 1.8 to 15 mg/L, within the MSC range. This is in contrast to the antimicrobial amoxicillin where hardly any residues were encountered, which is not surprising due to its instability.[15,35] One can argue that amoxicillin could be used instead of stable antimicrobials; however, amoxicillin is still used extensively and has selective properties for β-lactamase genes with possible co-selection for tetracycline resistance.[36,37] Nevertheless, persistence of antimicrobials should be taken into consideration in stewardship programmes as it extends the selection for phenotypically NWT *E. coli* (doxycycline approximately 52% increase, enrofloxacin approximately 100% increase). Moreover, increase of the occurrence of resistance genes after treatment with a stable antimicrobial compound (doxycycline; significance $P \leq 0.05$ and $P \leq 0.0001$) should also be considered. Therefore, to incorporate this in the antimicrobial classification would be valuable for the global efforts to encourage prudent usage of antimicrobials for reducing AMR, especially for livestock, where coprophagic behaviour is not uncommon.

Investigating the resistance levels phenotypically and genetically in relation to the stable residues gives a better understanding about prolonged selection for AMR bacteria due to recirculation of residues. Extensive research has been conducted into resistance levels in the farm environment[38–40] but to our knowledge not in combination with antimicrobial residual concentrations. Peng *et al.*[41] measured the antimicrobial concentrations of amoxicillin, doxycycline and ciprofloxacin (closely related to enrofloxacin) in manure during and after treatment of caged laying hens. In that study, birds were administered 50 mg/kg (among different concentrations) for all three antimicrobials, similar to our study. Peng *et al.* did find residual concentrations of amoxicillin after treatment; however, up to 2 days after the application they could not detect any concentrations in the group treated with 50 mg/kg. This is a slight difference compared with our study since we did not measure any concentrations of amoxicillin directly after treatment. Amoxicillin was probably degraded rapidly by the β-lactamase enzymes produced by the resistant bacteria or just by the general instability of the compound. This difference could be explained by the β-lactamase genes present in the microbiome. Peng *et al.* did not study the resistome, which could influence the amoxicillin concentration. Furthermore, in the study of Peng *et al.*, doxycycline and ciprofloxacin were measured with similar concentrations as we observed in our study (doxycycline 6 mg/kg and enrofloxacin 15 mg/kg). After the application time, Peng *et al.* did not detect any doxycycline after 7 days, which is unlike our findings as we measured residues after 37 days, possibly due to the usage of cages instead of pens. Furthermore, ciprofloxacin was measured with an average concentration of 5.78 mg/kg after terminating the treatment, comparable to our enrofloxacin measurements and the same trend as other studies determined.[42,43]

The objective of this study was to investigate if stable antimicrobial residues lead to increased AMR levels. The presence of amoxicillin resistance in the baseline measurement suggested existing resistance in the broilers, implying that there were already β-lactamase-producing bacteria present, despite our efforts to select offspring from a untreated parental flock. This might have influenced the results from the amoxicillin treatment group since we observed high amoxicillin resistance directly after the treatment, possibly because of direct selection of resistant bacteria. Another unexpected result was the higher levels of resistance in the resistome data at Day 37 for doxycycline and enrofloxacin. This can be explained as we used caecal material instead of faecal droppings. Caecal samples have been observed to have higher levels of resistance genes.[44] This is explaining the increased resistance levels compared with the control group despite the concentrations that are below the MSC at Day 37.

Considering the absence of resistance genes in the resistome after the enrofloxacin treatment, it can be argued that enrofloxacin does not select for resistance genes. Actually, this is described in the literature; resistance to enrofloxacin involves another selection mechanism based on selection or induction of SNPs associated with resistance instead of an increase in species carrying resistance genes.[45,46] We sequenced several of the enrofloxacin NWT *E. coli* strains from the enrofloxacin treatment where we observed SNPs responsible for enrofloxacin resistance (A. F. Swinkels, A. L. Zomer, J. A. Wagenaar, unpublished data).

Our experimental set-up is different from conditions of commercial broilers, due to smaller groups of animals and stricter hygiene protocols. Therefore, parameters would be fluctuating due to the differences in set-up and might influence the results generated from this research. However, we considered the density of the broilers as an essential parameter since this is important for

mimicking the commercial setting and enabling coprophagy. To conclude, we believe that persistence is an important factor in development of AMR.

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Transparency declarations

None to declare.

Supplementary data

Supplementary materials for this article may be found online at https://zenodo.org/records/11103906.

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