

Occurrence of tetracycline residues in cattle manure from southern Malawi: implications for antimicrobial resistance and One Health

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1 **Occurrence of tetracycline residues in cattle manure from southern Malawi: implications for**
2 **antimicrobial resistance and One Health**

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16

17 Abstract

18 Antibiotic residues in livestock manure are a recognized environmental driver of antimicrobial
19 resistance (AMR), yet no data exist on residue concentrations in cattle manure from Malawi despite
20 unregulated veterinary drug access, widespread sub-therapeutic dosing, and extensive use of manure
21 as agricultural fertiliser. We conducted a cross-sectional study in Chikwawa and Nsanje districts of
22 southern Malawi to quantify tetracycline-class residues in beef cattle manure by LC-MS/MS,
23 comparing intensive farm (n=49) and smallholder (n=41) production systems, with antimicrobial
24 susceptibility patterns of indicator Enterobacteriaceae evaluated to provide contextual evidence for
25 the environmental implications of residue contamination. Fresh manure samples (n=90) were
26 analysed for tetracycline-class residues and for Enterobacteriaceae AMR by Kirby-Bauer disk
27 diffusion. Chlortetracycline, tetracycline, and doxycycline were detected in all 90 samples;
28 oxytetracycline in 55 (61.1%). Chlortetracycline and tetracycline concentrations were significantly
29 higher in intensive farm samples (Mann-Whitney U test: $p=0.0009$ and $p<0.001$), consistent with
30 more systematic antibiotic use in commercial production. Indicator Enterobacteriaceae isolates
31 showed universal tetracycline resistance (100%; 95% CI 96.1–100.0%) and near-universal
32 amoxicillin-clavulanic acid resistance (96.7%; 95% CI 90.8–99.3%), alongside low resistance to
33 cefpodoxime (7.6%), ciprofloxacin (5.4%), and trimethoprim-sulphamethoxazole (4.7%), and no
34 gentamicin resistance; isolates resistant to all five tested antibiotics were exclusively from intensive
35 farm samples, though overall multidrug resistance (≥ 3 antimicrobial classes) did not differ
36 significantly between production systems. No significant correlation was detected between residue
37 concentrations and resistance proportions, consistent with resistance already being fixed at the
38 population level rather than driven by contemporaneous residue burden. In exploratory regression
39 modelling, female cattle had significantly higher doxycycline concentrations than males (4.75 vs.
40 4.30 ppb; $p=0.045$). These findings provide the first evidence of widespread tetracycline-class residue
41 contamination in cattle manure from Malawi, co-occurring with highly resistant Enterobacteriaceae,
42 and underscore the One Health risks of applying residue-laden manure to agricultural land,
43 highlighting an urgent need for antimicrobial stewardship policy, veterinary drug regulation, and
44 manure management practice.

45

46 Keywords: antibiotic residues; cattle manure; tetracyclines; antimicrobial resistance;
47 Enterobacteriaceae; Malawi; One Health

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50 Background

51 Antibiotic residues released into the environment via livestock manure are an increasingly recognised,
52 and comparatively understudied, environmental driver of antimicrobial resistance (AMR), one of the
53 top three global health challenges identified by the joint FAO/OIE/WHO Tripartite, with an estimated
54 700,000 deaths annually and projections of 10 million by 2050 if no action is taken [1, 2]. The One
55 Health framework underscores that this problem transcends disciplinary boundaries, existing at the
56 interface of human medicine, animal husbandry, and environmental reservoirs [1, 3]. Livestock
57 production is a key source of these residues: the widespread use of antibiotics for disease prevention,
58 treatment, and growth promotion means that a large proportion of administered compounds is
59 excreted unmetabolised, entering manure and, from there, the wider environment, where residual
60 concentrations can create selective pressure on bacterial communities long after treatment [4].

61 Animals are unable to fully metabolise administered antibiotics with up to 90% of compounds
62 excreted in urine or faeces as parent compounds, or active and inactive metabolites [4, 5]. This results
63 in high antibiotic residue concentrations in manure, ranging from traces to over 200 mg kg⁻¹ [6].
64 Global evidence documents this problem for example, in China, oxytetracycline (56 mg kg⁻¹),
65 chlortetracycline (28 mg kg⁻¹), enrofloxacin (47 mg kg⁻¹), and ciprofloxacin (30 mg kg⁻¹) have been
66 documented in cattle manure [7]; in the Netherlands, 75% of 646 cattle faecal samples tested positive
67 for residues including oxytetracycline, ciprofloxacin, and colistin [8]. Land application of residue-
68 laden manure disseminates ARGs into agricultural soils and leaches into groundwater, contaminating
69 the broader ecosystem [9, 10, 11], establishing a plausible exposure chain: antibiotics administered
70 to livestock are excreted as residues in manure; manure is applied to crop fields, where tetracyclines
71 can persist in tropical soils; this persistence selects for resistant bacteria within the soil and water
72 microbiota; and runoff or leaching can carry both residues and resistant organisms to surface water
73 and onto vegetables, creating routes of human exposure [9, 10, 11]. Enterobacteriaceae, including
74 critically important zoonotic pathogens such as *Salmonella* spp. and *Escherichia coli*, are recognised
75 indicator organisms for monitoring this process, given their established role as vehicles for multi-
76 drug resistance acquisition and inter-species ARG transfer [12].

77 In Malawi, there are no clear national policies governing the purchase or use of antibiotics in
78 livestock, resulting in unregulated access to veterinary drugs at retail level [13]. Studies have
79 documented that farmers in Malawi administer antibiotic mixtures containing colistin (a critical last-
80 resort antibiotic banned for livestock use) and that vitamin supplements routinely include low-dose
81 antibiotics, exposing animals to sub-therapeutic concentrations across multiple antibiotic classes
82 simultaneously [13]. Such sub-therapeutic, chronic exposure is a well-established driver of de novo
83 resistance mutations and selective amplification of resistant bacteria [14]. Cattle manure is also

84 extensively used as an agricultural fertiliser given the high cost of synthetic alternatives, creating a
85 direct pathway for ARGs to enter soils, surface water, and the shallow groundwater on which rural
86 communities depend [15, 16]. We hypothesized that antibiotic access and use practices would differ
87 between these two production types, with intensive commercial farms more likely to obtain veterinary
88 drugs through formal supply chains and administer them systematically, compared with smallholder
89 systems, which often rely on informal or shared drug sources. Comparing intensive and smallholder
90 systems was therefore expected to help identify where residue and resistance risks are concentrated
91 and where interventions would have the greatest impact. Tetracyclines are the most widely used
92 antibiotic class in Malawian livestock production and were therefore the primary target class for
93 residue analysis in this study [16, 17].

94 Despite this context, no published studies have quantified antibiotic residue concentrations in cattle
95 manure in Malawi, nor characterised the resistance profile of associated indicator bacteria. This
96 represents a critical evidence gap that impedes the development of evidence-based policies and
97 antimicrobial stewardship programmes. This study was guided by two a priori hypotheses: (1)
98 antibiotic residues from multiple classes would be detectable in manure from both production systems
99 but at different concentrations; and (2) the prevalence and profiles of AMR among indicator
100 Enterobacteriaceae would differ between intensive and smallholder systems, reflecting differences in
101 residue exposure. Accordingly, the study aimed to: (1) quantify tetracycline-class residues in beef
102 cattle manure by LC-MS/MS from intensive and smallholder production systems in Chikwawa and
103 Nsanje districts; (2) describe antimicrobial susceptibility patterns among indicator Enterobacteriaceae
104 to provide contextual evidence for the environmental AMR risks associated with residue
105 contamination; and (3) formally test whether residue concentrations and AMR profiles differ between
106 production systems and determine their statistical correlation. The findings are intended to serve as a
107 baseline for national residue and AMR surveillance and to inform the formulation of regulatory
108 frameworks for antibiotic use and manure management in food animal production in Malawi.

109 **Materials and Methods**

110 **Study design**

111 This cross-sectional pilot study was conducted March-June 2024 in Chikwawa and Nsanje districts
112 (Lower Shire valley) of southern Malawi. These districts were selected because the lower Shire valley
113 has one of the highest cattle population densities in Malawi and uniquely hosts both intensive
114 commercial farms and smallholder production systems in close geographic proximity, enabling a
115 direct comparison between the two production types. Sample size was calculated using the Cochran
116 formula assuming a 95% confidence level, 5% precision, and 50% estimated prevalence (used in the
117 absence of prior local data), yielding a minimum of 80 samples; 90 were collected to allow for
118 attrition.

119 **Sample collection**

120 Samples were collected per rectum in a crush pen to ensure fresh, uncontaminated material;
121 otherwise, fresh voided material was collected immediately upon defecation and placed into sealed
122 sterile plastic containers. Samples were stored in a cooler box with ice packs at 4°C and transported
123 within 4-6 h to the Kamuzu University of Health Sciences (KuHeS) Microbiology Laboratory in
124 Blantyre. Sequential identifiers (TK001-TK090) were assigned at collection and used consistently for
125 both bacteriological and residue analyses throughout the study. Upon arrival, samples were
126 homogenised; a 2 g aliquot was frozen at -20°C in polypropylene tubes for later chemical residue
127 analysis, while bacteriological analysis was done immediately.

128 **Bacteriological analyses**

129 Enterobacteriaceae were selected as indicator organisms for monitoring AMR in cattle manure,
130 consistent with common practice in environmental AMR surveillance; family-level confirmation was
131 therefore sufficient for the objectives of this residue-focused pilot study, and species-level
132 identification was not undertaken.

133 **Enumeration.** One gram of each sample was suspended in 9 mL buffered peptone water and serial
134 dilutions (10^{-1} – 10^{-4}) plated (100 μ L) in duplicate on VRBG agar (Violet Red Bile Glucose agar;
135 Oxoid, Thermo Fisher Scientific, UK; 37°C, 24 h). Pink and blue colonies are characteristic of
136 Enterobacteriaceae; white colonies indicate non-Enterobacteriaceae organisms. Too numerous to
137 count (TNTC plates) were recorded as such. Where both morphotypes were present, one
138 representative of each was subcultured, yielding up to two isolate records per sample.

139 **Confirmation.** Oxidase-negative colonies producing yellow colour in glucose OF medium under oil
140 overlay were confirmed as Enterobacteriaceae. Confirmed isolates were plated on MacConkey agar

(Oxoid, Thermo Fisher Scientific, UK) and Extended Spectrum Beta Lactamases (ESBL)-selective chromagar (CHROMagar ESBL, CHROMagar, Paris, France; supplemented with 25 mg/L cefotaxime).

Antimicrobial Susceptibility Testing. Isolates were tested by Kirby-Bauer disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines [CLSI M100] using the following antibiotic disks: amoxicillin-clavulanic acid (30 ug), tetracycline (30 ug), cefpodoxime (30 ug), ciprofloxacin (5 ug), gentamicin (10 ug), and trimethoprim-sulphamethoxazole (TMP-SMX, 30 ug). The selection of these antibiotics was based on their clinical importance and documented use in Malawian livestock production [13, 17]: tetracyclines are the most widely administered class in Malawian farms; fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin) are critically important human antibiotics; beta-lactams (amoxicillin-clavulanic acid, cefpodoxime) and sulfonamides (TMP-SMX) are essential for both veterinary and human medicine. After 24 h incubation, zones of inhibition were measured and interpreted as Susceptible (S), Intermediate (I), or Resistant (R) based on published breakpoints [20]. Where results could not be obtained, isolates were recorded as ND (not done). Multidrug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial classes, per the international expert consensus definition [16].

158

Antibiotic residue analysis

Analysis was performed at the Malawi Bureau of Standards (MBS) laboratory in Blantyre by LC-MS/MS under two analytical reports: PES/24/BA 17 (TK001-TK050; four compounds: chlortetracycline, tetracycline, doxycycline, oxytetracycline) and PES/24/BA 24 (TK051-TK090; three compounds: chlortetracycline, tetracycline, doxycycline only). The multi-residue extraction protocol was adapted from van den Meersche and colleagues [18]: 2 mg of homogenised suspension was dissolved in 10 mL water/acetonitrile (80:20 v/v), shaken for 30 min on a horizontal shaker, centrifuged at 4000 x g for 15 min at 20° C, and the supernatant evaporated to dryness at 45° C. The residue was reconstituted in 1 mL water/acetonitrile (80:20) with 0.1% formic acid, vortex-mixed, sonicated for 5 min, and filtered through a 0.22 um membrane. The extract was diluted 1:10 and re-filtered before injection. Analyses were performed on an Agilent 1260 binary HPLC coupled to an Agilent 6410 Triple Quadrupole mass spectrometer using a HyPURITY C18 column (250 x 2.1 mm ID, 5 um particle size) at 50° C. Results are reported in ppb (ug/kg wet weight). Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3x(SD/S) and 10x(SD/S) respectively, where SD is the standard deviation of y-intercepts of calibration regression lines and S is the slope.

174 **Statistical analysis**

175 All statistical analyses were performed in R (v4.3) using the packages dplyr v1.0.7 (data
176 manipulation), reshape2 v1.4.4 (data reshaping), and ggplot2 v3.3.5 (visualisation) [17, 18, 19]. Non-
177 parametric Mann-Whitney U tests were used to assess between-group differences in residue
178 concentrations by production setting (Farm vs. Individual Farmer) and by district (Chikwawa vs.
179 Nsanje), as residue data were right-skewed. Oxytetracycline was excluded from between-setting
180 comparisons due to unbalanced analyte panel coverage across MBS batches. Fisher's exact tests were
181 used to compare categorical AMR prevalence proportions between production settings and districts,
182 with a significance threshold of $\alpha=0.05$; Fisher's exact test was chosen over Chi-square because
183 several resistance outcomes (cefpodoxime, ciprofloxacin, TMP-SMX) had expected cell counts
184 below five, for which Fisher's exact test is the appropriate method. To formally investigate the
185 hypothesised relationship between residue burden and resistance, Spearman rank correlations were
186 computed between residue concentrations (chlortetracycline, tetracycline, doxycycline) and
187 resistance proportions at the sample level ($n=70$ paired samples) for four AMR outcomes; Benjamini-
188 Hochberg false discovery rate correction was applied to address multiple comparisons. Conditional
189 density plots were generated to visualise how resistance phenotypes were distributed across the range
190 of observed residue concentrations. Principal component analysis (PCA) was performed on the
191 standardised combined residue and resistance matrix ($n=70$) to explore multivariate structure. Binary
192 logistic regression modelled the probability of resistance as a function of standardised residue
193 concentrations for outcomes with sufficient variation (cefpodoxime, ciprofloxacin, TMP-SMX);
194 model fit was assessed by likelihood ratio test. Given the small number of resistant outcomes per
195 model (4-7 events), logistic regression results are reported as entirely exploratory and hypothesis-
196 generating only. To identify predictors of tetracycline-class residue concentrations, three separate
197 multivariable linear regression models were fitted, one per outcome: log-transformed
198 chlortetracycline, tetracycline, and doxycycline concentrations ($n=90$). Log-transformation was
199 applied to address the right-skewed distribution of residue data. Each model included production
200 setting (Farm vs. Individual Farmer, reference), district (Nsanje vs. Chikwawa, reference), and animal
201 sex (Male vs. Female, reference) as predictors. Regression coefficients (β) represent the estimated
202 difference in log-residue concentration per unit change in each predictor. Overall model fit was
203 assessed by F-test and R^2 . Ninety-five percent confidence intervals for regression coefficients were
204 calculated from the standard errors of the ordinary least squares estimates; confidence intervals for
205 resistance prevalence proportions were calculated using the Wilson score method.

206 **Results**

207 **Study population**

208 A total of 90 manure samples were collected: 63 from Chikwawa (22 Farm, 41 Individual Farmer)
209 and 27 from Nsanje (all Farm). Sex was recorded as follows 62 bulls and 28 cows (Farm: 28 bulls,
210 21 cows; Individual Farmer: 34 bulls, 7 cows; Chikwawa: 55 bulls, 8 cows; Nsanje: 7 bulls, 20 cows).
211 Of 90 samples, 70 yielded culture-positive (77.8%) and 20 culture-negative (22.2%) for
212 Enterobacteriaceae. Of the 70 culture-positive samples, 42 produced pink colonies only, 3 blue only,
213 24 both pink and blue morphotypes (6 also producing white colonies), and 1 produced blue and white
214 colonies. On VRBG agar, pink colonies indicate lactose-fermenting Enterobacteriaceae,
215 (presumptive *Escherichia coli* and *Klebsiella* spp.) and blue colonies indicate non-lactose-fermenting
216 Enterobacteriaceae (presumptive *Salmonella* or *Proteus* spp.); white colonies represent non-
217 Enterobacteriaceae organisms and were not sub-cultured. Isolates were confirmed to family level
218 (Enterobacteriaceae) by oxidase-negative and glucose OF-positive results. Enterobacteriaceae were
219 treated as indicator organisms for monitoring AMR in this residue-focused pilot study; species-level
220 identification was therefore not performed, consistent with this study design. TNTC MacConkey
221 growth was recorded for all 70 culture-positive samples. ESBL chromagar growth was observed in 6
222 of 70 culture-positive samples (8.6%). All 70 culture-positive samples contributed AST records; 22
223 samples contributed two records each and 48 one record each, yielding 92 total isolate records (56
224 Farm, 36 Individual Farmer). Results were not obtained (ND) for TMP-SMX in 7 of these isolates
225 due to insufficient bacterial lawn growth on re-subculture, which precluded valid zone measurement;
226 all other antibiotic disks were tested across all 92 isolates.

227 **Antibiotic residue concentrations**

228 Chlortetracycline, tetracycline, and doxycycline were detected in all 90 samples with Oxytetracycline
229 in 55 samples. No below-LOD results were recorded wherever tested. Chlortetracycline showed the
230 greatest variability (SD 13.60 ppb, maximum 111.9 ppb). Tetracycline also showed high variability
231 (SD 6.75 ppb, peak 57.6 ppb). Doxycycline was uniformly distributed (range 2.2–7.4 ppb) and
232 oxytetracycline was low and consistent (range 2.0–7.2 ppb) (Table 1).

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238 **Table 1. Residue descriptive statistics.**

Compound	n	Mean (ppb)	SD	Median	Min	Max
Chlortetracycline	90	7.36	13.60	4.80	4.7	111.9
Tetracycline	90	4.97	6.75	3.30	2.9	57.6
Doxycycline	90	4.44	1.23	4.35	2.2	7.4
Oxytetracycline*	55	3.14	1.10	2.70	2.0	7.2

239 * TK001–TK055 only (MBS Report PES/24/BA 17).

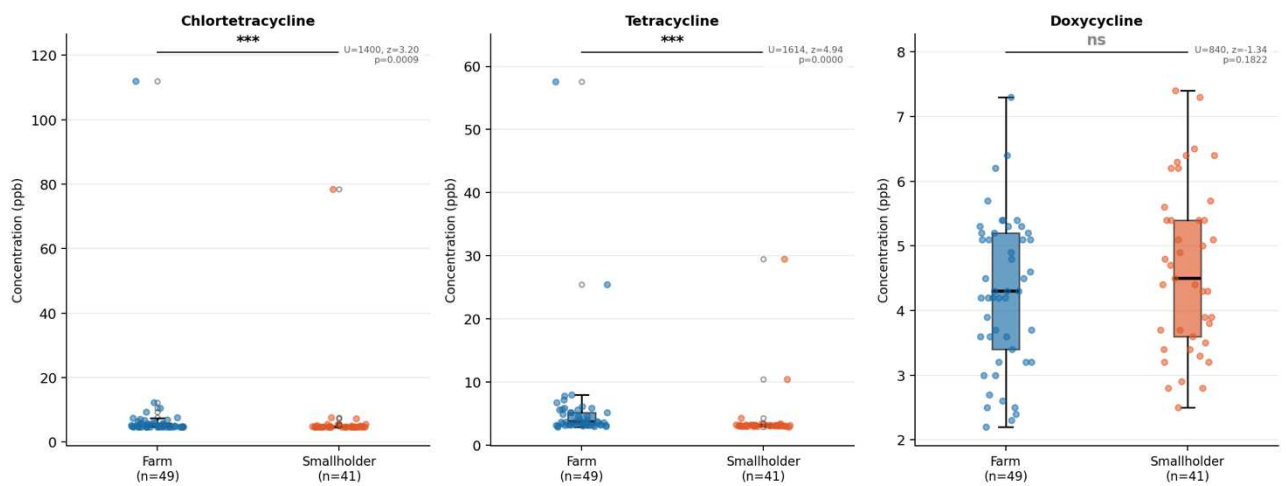
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241 **Residue concentrations by production setting**

242 Chlortetracycline was significantly higher in Farm (mean 7.84 ppb, SD 15.25, median 4.90) than
243 Individual Farmer samples (mean 6.79 ppb, SD 11.48, median 4.80; U=1400, z=3.20, p=0.0009) and
244 tetracycline significantly higher in Farm (mean 5.77, SD 8.25, median 3.70) than Individual Farmer
245 samples (mean 4.01, SD 4.24, median 3.20; U=1614, z=4.94, p<0.001). Doxycycline did not differ
246 significantly (p=0.182). Oxytetracycline was not compared between settings due to unbalanced
247 analyte coverage (Farm n=14, Individual Farmer n=41 within the 55 tested) (Figure 1).

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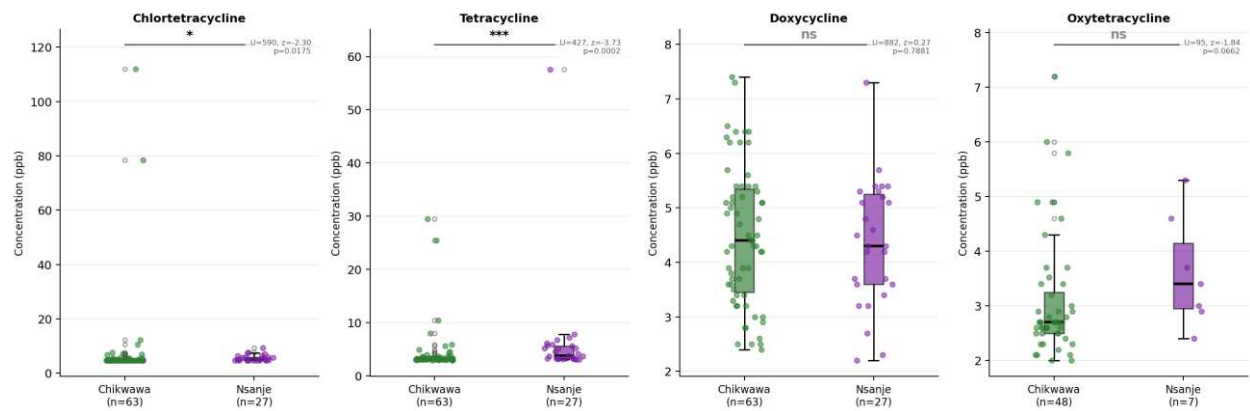
251 Figure 1. Tetracycline-class residue concentrations by production setting. Boxplots with jittered
252 data. Significance: *** p<0.001; ns=not significant (Mann-Whitney U). U and z statistics shown
253 within each panel.

254 **Residue concentrations by district**

255 Chlortetracycline was significantly higher in Chikwawa (mean 8.11, SD 16.22, n=63) than Nsanje
256 (mean 5.61, SD 1.14, n=27; U=590, z=-2.30, p=0.018). Tetracycline was significantly higher in

257 Nsanje (mean 6.44, SD 10.32) than Chikwawa (mean 4.33, SD 4.41; U=427, z=-3.73, p=0.0002).
 258 Doxycycline did not differ significantly (p=0.788). Oxytetracycline comparison is cautious
 259 (Chikwawa mean 3.07, n=48; Nsanje mean 3.61, n=7; p=0.066; ns) (Figure 2).

260



261

262 Figure 2. Tetracycline-class residue concentrations by district. Boxplots with jittered data.

263 Significance: *** p<0.001; * p<0.05; ns=not significant. U and z statistics shown within each panel.

264 **Predictors of residue concentration**

265 Multivariable linear regression models including production setting, district, and animal sex as
 266 predictors explained limited variance in residue concentrations across all three outcomes (Table 2).
 267 No overall model reached conventional significance (chlortetracycline: R²=0.026, F(3,86)=0.778,
 268 p=0.509; tetracycline: R²=0.077, F(3,86)=2.388, p=0.074; doxycycline: R²=0.072, F(3,86)=2.212,
 269 p=0.093). Production setting and district were not significant predictors for any compound after
 270 mutual adjustment with sex.

271 Animal sex was the only significant individual predictor: male cattle had significantly lower log-
 272 doxycycline concentrations than female cattle (β =-0.167, 95% CI -0.330 to -0.004, p=0.045),
 273 corresponding to lower mean doxycycline concentrations in males (mean 4.30 ppb, SD 1.19)
 274 compared to females (mean 4.75 ppb, SD 1.26). No other predictor reached significance in any
 275 model (Table 2). These findings are exploratory given the low R² values and the absence of overall
 276 model significance.

277 **Table 2. Multivariable linear regression models for predictors of log-transformed**
 278 **tetracycline-class residue concentrations (n=90).**

Outcome	Predictor	β (95% CI)	p	R ²	F p
<i>log(Chlortetracycline)</i>					

	Setting (Farm)	0.167 (−0.080 to 0.415)	0.182	0.026	0.509
	District (Nsanje)	−0.092 (−0.416 to 0.233)	0.577		
	Sex (Male)	0.067 (−0.201 to 0.335)	0.620		
<i>log(Tetracycline)</i>					
	Setting (Farm)	0.191 (−0.063 to 0.445)	0.139	0.077	0.074
	District (Nsanje)	0.085 (−0.249 to 0.420)	0.613		
	Sex (Male)	−0.055 (−0.331 to 0.220)	0.690		
<i>log(Doxycycline)</i>					
	Setting (Farm)	−0.095 (−0.245 to 0.056)	0.214	0.072	0.093
	District (Nsanje)	−0.063 (−0.261 to 0.134)	0.525		
	Sex (Male)	−0.167 (−0.330 to −0.004)	0.045 *		

279 β = regression coefficient on log-transformed residue concentration. 95% CI calculated from
 280 ordinary least squares standard errors. Reference categories: Individual Farmer (setting), Chikwawa
 281 (district), Female (sex). R^2 = proportion of variance explained; F p = overall model F-test p-value. *
 282 $p < 0.05$ (bold, yellow shading). All 90 samples had complete data for all predictors.

284 Near-universal resistance to tetracyclines and amoxicillin-clavulanic acid Universal tetracycline
 285 resistance (100%; 92/92; 95% CI 96.1–100.0%) and near-universal amoxicillin-clavulanic acid
 286 resistance (96.7%; 89/92; 95% CI 90.8–99.3%) were the dominant phenotypes. Cefpodoxime (7.6%;
 287 7/92; 95% CI 3.1–15.0%), ciprofloxacin (5.4%; 5/92; 95% CI 1.8–12.2%), and TMP-SMX (4.7%;
 288 4/85; 95% CI 1.3–11.6%) resistance was low. Gentamicin resistance was absent (0/92; 95% CI 0.0–
 289 3.9%) (Table 3; Figure 3)

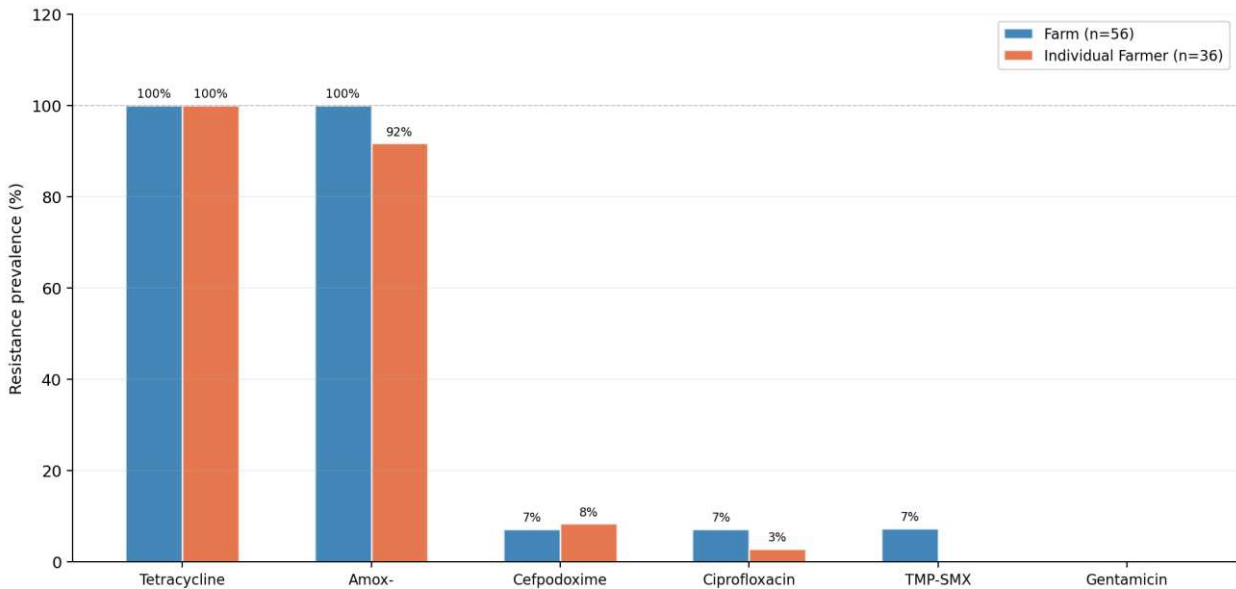
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298 **Table 3. Antimicrobial resistance prevalence (n=92 isolates).**

Antibiotic	Category	Tested	R	R (%)	95% CI	I	ND
Tetracycline	Tetracycline	92	92	100.0	96.1–100.0%	0	0
Amoxicillin- Clavulanic Acid	Beta-lactam/BLI	92	89	96.7	90.8–99.3%	0	0
Cefpodoxime	Cephalosporin	92	7	7.6	3.1–15.0%	0	0
Ciprofloxacin	Fluoroquinolone	92	5	5.4	1.8–12.2%	0	0
TMP-SMX	Sulfonamide	85	4	4.7	1.3–11.6%	0	7
Gentamicin	Aminoglycoside	92	0	0.0	0.0–3.9%	0	0

299 R=Resistant; I=Intermediate; ND=Not Done. TMP-SMX denominator excludes 7 ND records.

300



301

302 Figure 3. AMR prevalence by production setting (n=92). Values above bars show resistance
303 percentage.

304 No significant between-setting differences in individual antibiotic resistance prevalence Fisher's
305 exact tests showed no statistically significant differences between Farm and Individual Farmer
306 isolates for any antibiotic (Table 4). Amoxicillin-clavulanic acid resistance trended higher in Farm
307 isolates (100% vs. 91.7%; p=0.057). MDR: 5/56 Farm (8.9%) vs. 4/36 Individual Farmer (11.1%);
308 p=0.733.

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312 **Table 4. AMR by production setting (Fisher's exact test).**

Antibiotic	Farm n/N (%)	Ind. Farmer n/N (%)	p-value	Sig.
Tetracycline	56/56 (100.0)	36/36 (100.0)	1.000	ns
Amoxicillin-Clavulanic Acid	56/56 (100.0)	33/36 (91.7)	0.057	ns
Cefpodoxime	4/56 (7.1)	3/36 (8.3)	1.000	ns
Ciprofloxacin	4/56 (7.1)	1/36 (2.8)	0.645	ns
TMP-SMX	4/55 (7.3)	0/30 (0.0)	0.292	ns
Gentamicin	0/56 (0.0)	0/36 (0.0)	1.000	ns
MDR (≥ 3 classes)	5/56 (8.9)	4/36 (11.1)	0.733	ns

313 ns=not significant. TMP-SMX denominator excludes 7 ND records.

314

315 AMR prevalence did not differ significantly by district No statistically significant differences were found
 316 by district for any antibiotic (Table 5). AMC resistance: 94.9% Chikwawa vs. 100% Nsanje
 317 (p=0.550). CEF resistance: 10.2% vs. 3.0% (p=0.415).

318 **Table 5. AMR by district (Fisher's exact test).**

Antibiotic	Chikwawa n/N (%)	Nsanje n/N (%)	p-value	Sig.
Tetracycline	59/59 (100.0)	33/33 (100.0)	1.000	ns
Amoxicillin-Clavulanic Acid	56/59 (94.9)	33/33 (100.0)	0.550	ns
Cefpodoxime	6/59 (10.2)	1/33 (3.0)	0.415	ns
Ciprofloxacin	4/59 (6.8)	1/33 (3.0)	0.651	ns
TMP-SMX	3/52 (5.8)	1/33 (3.0)	1.000	ns
Gentamicin	0/59 (0.0)	0/33 (0.0)	1.000	ns

319 ns=not significant.

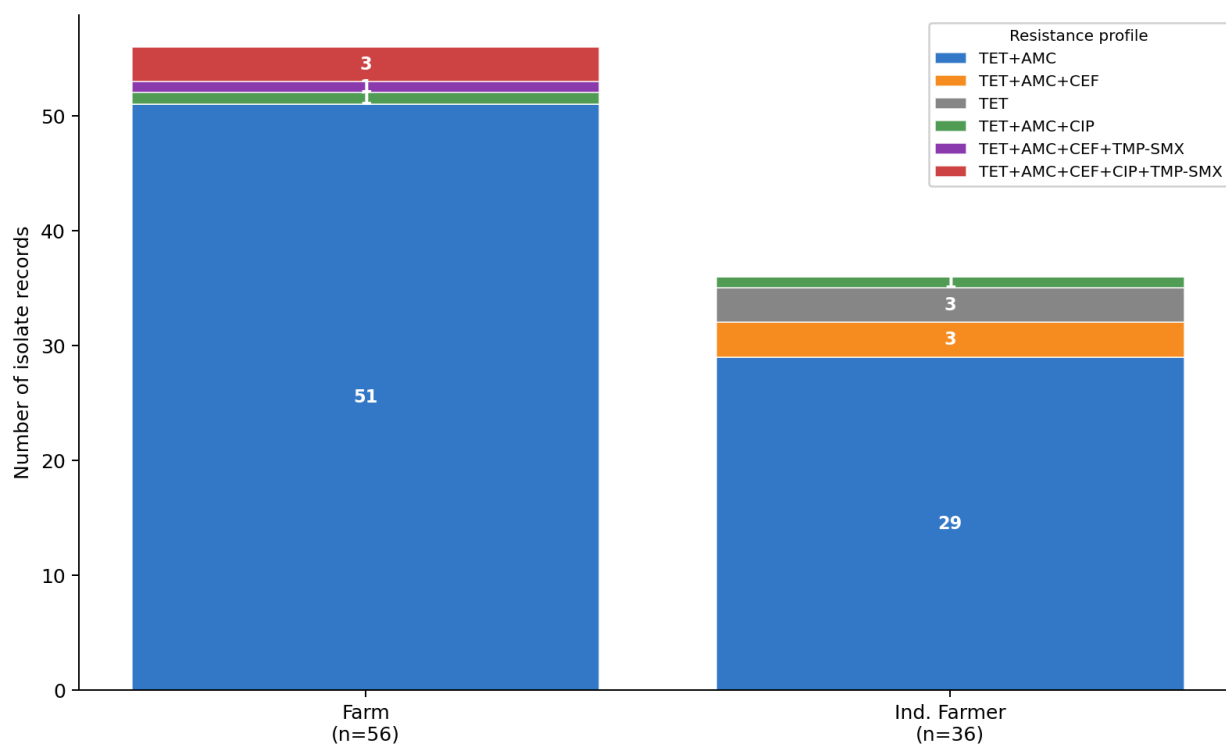
320

321 TET+AMC dual resistance dominant across both settings; pan-resistant isolates (resistant to all five
 322 tested antibiotics) exclusive to intensive farm isolates TET+AMC dual resistance dominated (80/92
 323 records, 87.0%; Table 6), representing the most prevalent co-resistance pattern across both production
 324 settings. This profile was observed in 51 of 56 Farm isolates (91.1%) and 29 of 36 Individual Farmer
 325 isolates (80.6%), as illustrated in Figure 4. Three records, all from Farm isolates, were resistant to all
 326 five tested antibiotics (TET+AMC+CEF+CIP+TMP-SMX; hereafter "pan-resistant"), indicating that

the most extensive resistance phenotypes are concentrated in the intensive production setting. This is distinct from the broader MDR definition used in Table 4 (non-susceptibility to ≥ 3 antimicrobial classes), which also captures the TET+AMC+CEF and TET+AMC+CIP profiles below; under that broader definition, MDR prevalence did not differ significantly between settings (5/56 Farm, 8.9%, vs. 4/36 Individual Farmer, 11.1%; $p=0.733$). TET-only resistance (i.e., resistance to tetracycline alone without co-resistance to beta-lactams) was observed in 3 records, all from Individual Farmer isolates, suggesting that a subset of smallholder isolates has not yet accumulated beta-lactam co-resistance. The isolate-level heatmap (Figure 5) displays the complete resistance profiles for all 92 records, sorted by production setting, allowing visual identification of cluster patterns within Farm and Individual Farmer groups; rows represent individual isolate records and are coloured red (Resistant), yellow (Intermediate), green (Susceptible), or grey (Not Done). The clear vertical stripe of tetracycline and amoxicillin-clavulanic acid resistance across virtually all rows confirms the near-universal dual resistance phenotype, while the sparse red cells for cefpodoxime, ciprofloxacin, and TMP-SMX reflect the low but present secondary resistance.

Table 6. Resistance profiles by production setting (n=92 isolate records).

Resistance Profile	Total n (%)	Farm (n=56)	Ind. Farmer (n=36)
TET+AMC	80 (87.0%)	51	29
TET+AMC+CEF	3 (3.3%)	0	3
TET	3 (3.3%)	0	3
TET+AMC+CEF+CIP+TMP-SMX (full MDR)	3 (3.3%)	3	0
TET+AMC+CIP	2 (2.2%)	1	1
TET+AMC+CEF+TMP-SMX	1 (1.1%)	1	0
Total	92 (100%)	56	36



343

344 Figure 4. Resistance profiles by production setting. Numbers within bars = isolate record counts.

345 TET=tetracycline; AMC=amoxicillin-clavulanic acid; CEF=cefepodoxime; CIP=ciprofloxacin.

346

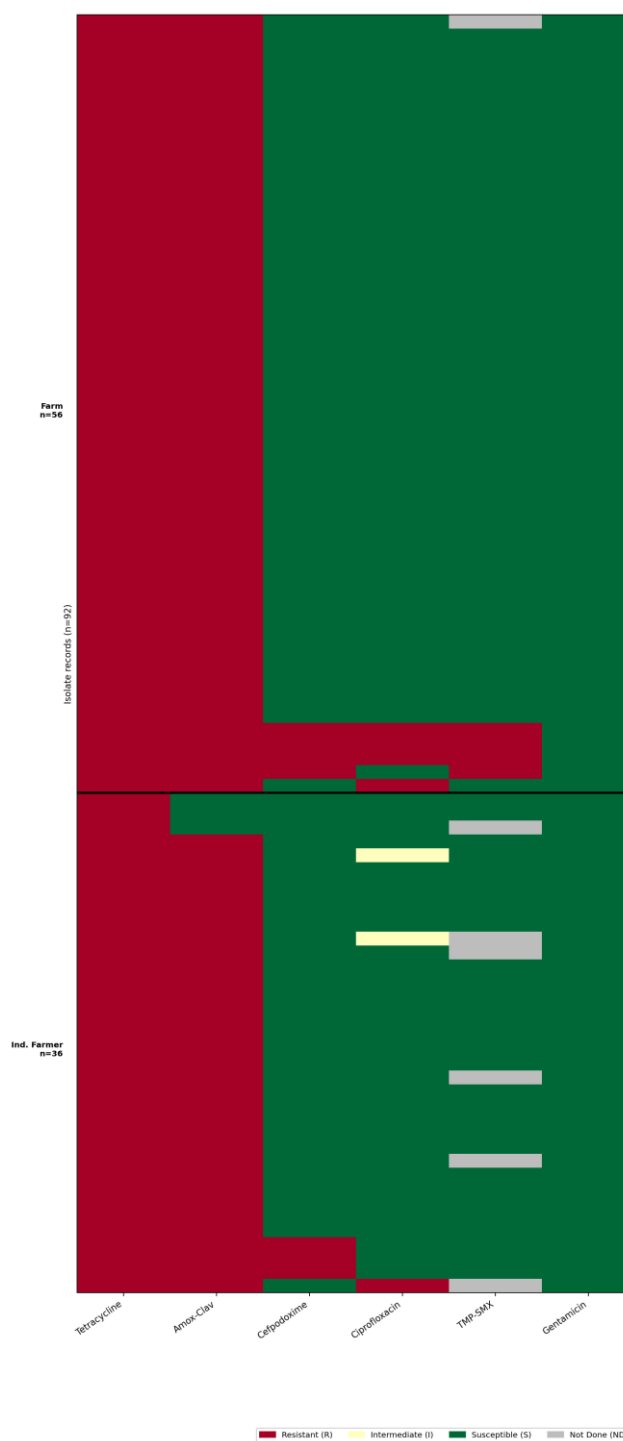


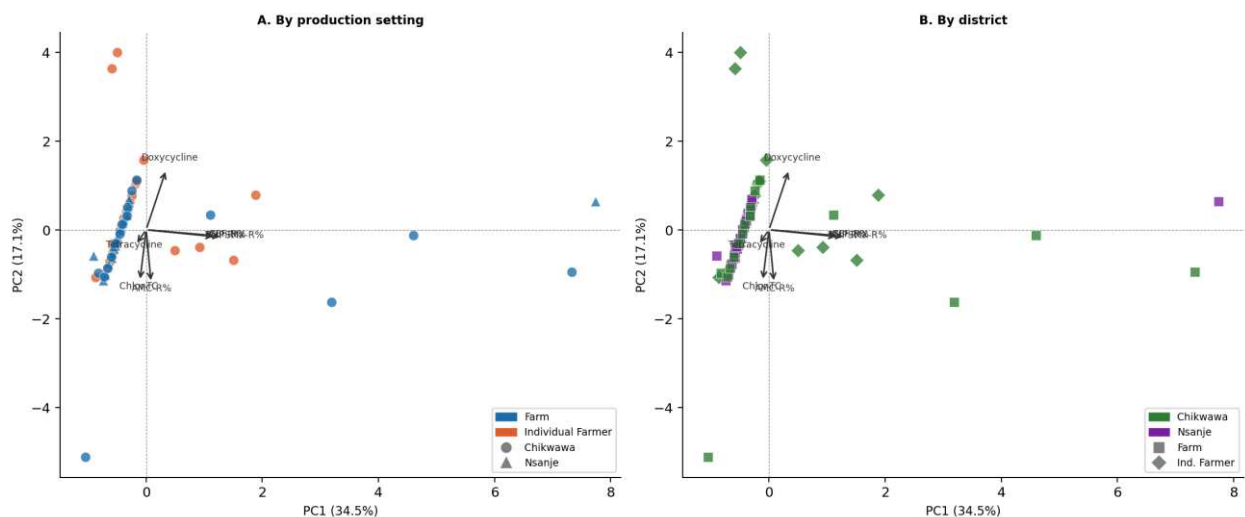
Figure 5. Isolate-level AMR heatmap (n=92 records). Sorted by setting and stratified by resistance profile. Red=Resistant; Yellow =Intermediate; Green=Susceptible; Grey=Not done. Black line separates Farm from Individual Farmer records (n=92 total rows; each row = one isolate record). Light-grey horizontal lines delineate individual rows. Grey (Not done) for TMP-SMX in 7 isolates reflects insufficient bacterial lawn growth on subculture precluding valid zone measurement.

355 **Residue–resistance correlations**

356 No significant Spearman correlation was detected for any of 12 compound–outcome combinations
357 after Benjamini-Hochberg correction (all adjusted $p > 0.05$; S1 Table and S1 Fig). The highest
358 observed value was chlortetracycline vs. AMC resistance ($\rho = 0.200$, $p = 0.097$). All non-significant
359 after Benjamini-Hochberg FDR correction.

361 **Principal Component Analysis (PCA)**

362 PCA on the combined standardised matrix of residue concentrations and resistance proportions ($n = 70$
363 paired samples) explained 34.5% of variance on PC1 and 17.1% on PC2 (Figure 6). No clear
364 separation was observed between Farm and Individual Farmer samples or between districts. AMC
365 resistance contributed minimally, consistent with its near-universal prevalence providing no
366 discriminatory information.



369 Figure 6. PCA biplot of combined residue and resistance data ($n = 70$ paired samples). Arrows
370 indicate loading directions; arrow length is proportional to the contribution of each variable to the
371 displayed principal components. Ellipses represent 95% confidence regions for each group. A:
372 coloured by production setting; B: coloured by district.

373 **Binary logistic regression and conditional density analysis**

374 The goal of this exploratory analysis was to formally test whether tetracycline-class residue
375 concentrations (chlortetracycline, tetracycline, and doxycycline, each standardised to unit variance)
376 independently predicted resistance to antibiotics with sufficient phenotypic variation (i.e., not
377 tetracycline or AMC, which showed near-universal resistance). Three separate binary logistic
378 regression models were fitted, one per outcome: cefpodoxime resistance ($n = 70$, $R = 7$), ciprofloxacin

379 resistance (n=70, R=5), and TMP-SMX resistance (n=70, R=4). Each model included only the three
380 standardised residue concentrations as predictors; no additional metadata (e.g., production setting,
381 district, sex) were included as covariates in this initial analysis given the very small event counts,
382 which precluded reliable estimation of additional parameters. No model achieved statistical
383 significance: cefpodoxime $\chi^2(3)=3.057$, $p=0.383$; ciprofloxacin $\chi^2(3)=2.370$, $p=0.499$; TMP-SMX
384 $\chi^2(3)=2.778$, $p=0.427$. Conditional density plots (S2 Fig) confirmed substantial overlap in residue
385 distributions between resistant and susceptible isolates for all combinations, with flat logistic
386 probability curves throughout the observed range. The forest plot (S3 Fig) shows wide confidence
387 intervals reflecting small event counts (4–7 per model). These results are entirely exploratory.

388

389

390

391 Discussion

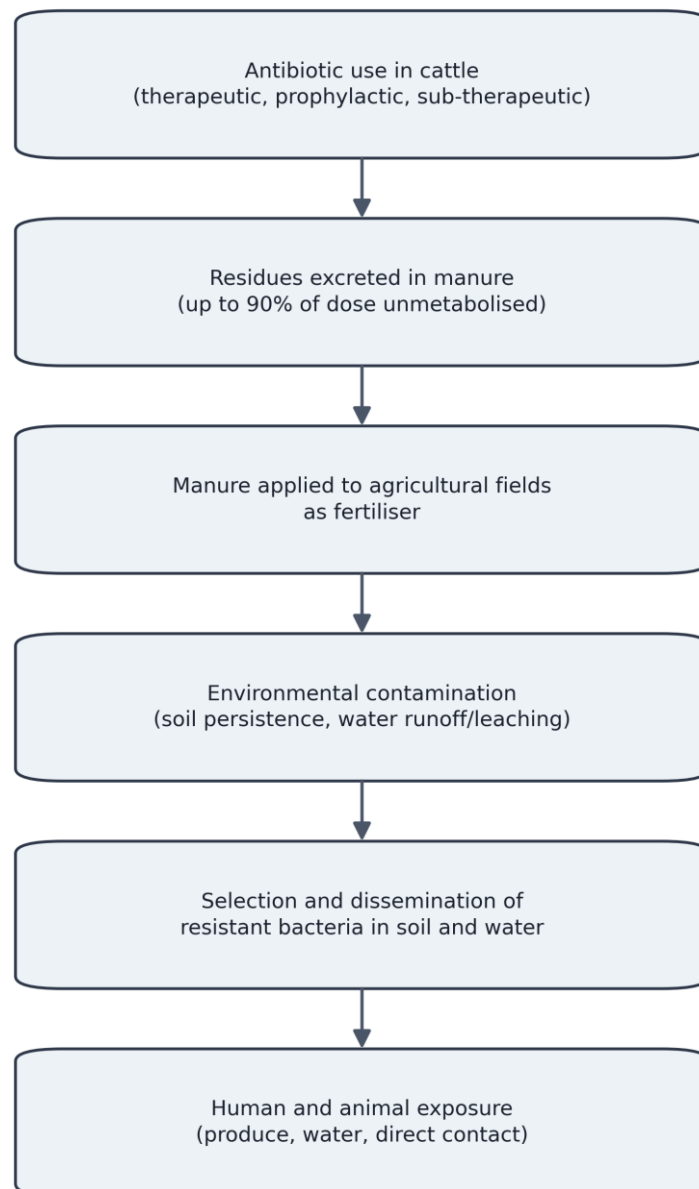
392 This study presents the first data on tetracycline-class antibiotic residues in beef cattle manure from
393 Malawi, with antimicrobial susceptibility patterns of indicator Enterobacteriaceae characterised to
394 provide contextual evidence for the environmental implications of residue contamination. Residues
395 were detectable in both settings but at significantly higher concentrations on intensive farms
396 (chlortetracycline and tetracycline; Mann–Whitney U test: $p=0.0009$ and $p<0.001$), while AMR
397 profiles were broadly similar between systems overall: pan-resistant isolates (resistant to all five
398 tested antibiotics) were exclusively from Farm samples, but overall MDR prevalence and near-ceiling
399 resistance to individual antibiotics did not differ significantly between settings, leaving little
400 discriminatory variance. Together, these findings establish a baseline for national residue and AMR
401 surveillance in Malawian livestock.

402 All tetracycline-class compounds were detected in every sample, confirming widespread exposure
403 across both production systems. Mean concentrations were low relative to settings with heavier
404 antibiotic use, such as Chinese feedlot manure [7], but consistent with other East and Central African
405 cattle studies [22, 23]. Higher concentrations on Farm samples reflect more systematic antibiotic use
406 in commercial settings, consistent with regional patterns [24, 25], while production setting and district
407 together explained limited variance in residue concentrations overall (R^2 2.6–7.7%) [13].

408 Tetracycline resistance (100%) and amoxicillin-clavulanic acid resistance (96.7%) substantially
409 exceed regional benchmarks, such as 10.6% tetracycline resistance in Zambian dairy cattle *E. coli*
410 [26], likely reflecting decades of unregulated, high-volume tetracycline use and sub-therapeutic
411 dosing [14, 16, 17]. By contrast, low resistance to cefpodoxime, ciprofloxacin, and TMP-SMX, and

412 the complete absence of gentamicin resistance, are reassuring and warrant protection through
413 stewardship policy [21]. Dual TET+AMC resistance (87.0%) is consistent with co-selection under
414 intensive antibiotic use [27, 28].

415 No significant correlation was detected between residue concentrations and AMR outcomes after
416 correction for multiple comparisons, most likely because resistance is already near-universally fixed,
417 leaving little outcome variance to explain, and because the logistic regression models were
418 underpowered. This null result does not negate the long-term role of tetracycline use in shaping
419 resistance, but reflects the limits of cross-sectional design once resistance is already fixed. The co-
420 occurrence of resistant Enterobacteriaceae and tetracycline residues in manure that is routinely
421 applied, untreated, to agricultural fields nonetheless represents a tangible One Health risk:
422 tetracyclines can persist in tropical soils for weeks to months [9, 10], and conventional practices here,
423 open storage followed by direct field spreading, do not reliably degrade residues or eliminate resistant
424 bacteria [27]; sustained thermophilic composting could substantially reduce both and is worth piloting
425 locally. Left untreated, land application creates a direct pathway for residues and resistant organisms
426 to reach the shallow groundwater on which rural communities depend [9, 10, 11], and could limit
427 first-line treatment options should resistant organisms reach humans. Routine residue monitoring,
428 veterinary drug regulation, and stewardship policy would together address this pathway (Figure 7).



429

430 Figure 7. Conceptual One Health pathway linking antibiotic use in cattle to environmental and
 431 human exposure. Residues excreted in manure persist through land application and environmental
 432 contamination, creating conditions that select for and disseminate resistant *Enterobacteriaceae*,
 433 which can ultimately reach humans and animals through contaminated produce, water, or direct
 434 contact.

435 These findings have practical implications for how antimicrobial stewardship is implemented in
 436 Malawi. Because intensive and smallholder systems access veterinary drugs through different
 437 channels, a single regulatory approach is unlikely to be effective across both: intensive farms, which
 438 obtain antibiotics through formal supply chains, are more directly reachable through prescription-
 439 only policies and veterinary oversight, whereas smallholder systems, which often rely on informal or
 440 shared drug sources [13], may require complementary approaches such as community animal health

441 worker training, farmer extension programmes, and stricter control at the point of retail sale. Given
442 that tetracycline resistance was already near-universal across both settings, stewardship efforts
443 focused solely on reducing current antibiotic use are unlikely to reverse existing resistance in the
444 short term; they are nonetheless essential to prevent further escalation and to protect antibiotics, such
445 as gentamicin, that remain effective and are classified as critically important for human medicine
446 [21]. Embedding residue and resistance monitoring into Malawi's existing livestock and public health
447 surveillance systems would allow these baseline findings to be tracked over time and would help
448 determine whether targeted interventions are having the intended effect.

449 **Limitations.** This cross-sectional pilot study has important limitations. Sample size limits statistical
450 power for multivariable analyses. Enterobacteriaceae were intentionally treated as indicator
451 organisms for AMR monitoring, consistent with environmental AMR surveillance practice; species-
452 level identification and molecular characterisation of resistance genes were beyond this study's
453 scope, though their absence precludes inference about specific determinants or zoonotic risk from
454 individual pathogens such as *Salmonella* or *E. coli*. Oxytetracycline was not quantified in 35 of 90
455 samples due to differing analyte panels between MBS batches (PES/24/BA 17 vs PES/24/BA 24).
456 The single sampling time point (March–June 2024) prevents assessment of seasonal variation, and
457 individual animal treatment records were not collected, limiting interpretation of the sex-
458 doxycycline association identified in regression modelling. Future studies should collect individual
459 treatment histories, incorporate whole-genome sequencing or targeted ARG PCR panels, and
460 include seasonal repeat sampling and species-level identification to build on this baseline.

461

462 **Conclusions**

463 This study provides the first quantitative evidence of tetracycline-class antibiotic residues in cattle
464 manure from Malawi and documents their co-occurrence with highly resistant indicator
465 Enterobacteriaceae. Chlortetracycline and tetracycline concentrations were significantly higher in
466 intensive farm samples, though production setting and district together explained limited variance in
467 residue concentrations overall. As contextual evidence for the environmental implications of residue
468 contamination, Enterobacteriaceae exhibited near-universal tetracycline and amoxicillin-clavulanic
469 acid resistance across both settings; isolates resistant to all five tested antibiotics occurred exclusively
470 in intensive farm samples, though overall MDR prevalence did not differ significantly between
471 production systems; and gentamicin resistance was absent. No significant correlation was detected
472 between residue concentrations and resistance proportions, consistent with resistance already being
473 fixed at the population level rather than driven by contemporaneous residue burden. Exploratory
474 regression modelling identified female sex as a significant predictor of higher doxycycline
475 concentrations, a novel finding that warrants investigation in larger studies with individual animal
476 treatment records. Collectively, these findings establish a baseline for national residue and AMR
477 surveillance in Malawian livestock and support: (1) regulatory frameworks governing veterinary drug
478 access and manure management, to limit the volume of residues entering agricultural soils and water;
479 (2) antimicrobial stewardship programmes targeting tetracycline and beta-lactam use in both
480 intensive and smallholder cattle production; and (3) larger longitudinal studies incorporating whole-
481 genome sequencing, individual treatment records, and seasonal repeat sampling to characterise
482 resistance determinants and residue predictors more robustly.

483 **Declarations**

484 **Abbreviations**

485 The following abbreviations are used throughout this manuscript: antimicrobial resistance (AMR),
486 amoxicillin-clavulanic acid (AMC), antimicrobial resistance genes (ARGs), antimicrobial
487 susceptibility testing (AST), confidence interval (CI), Clinical and Laboratory Standards Institute
488 (CLSI), extended-spectrum beta-lactamase (ESBL), Food and Agriculture Organization of the United
489 Nations (FAO), false discovery rate (FDR), liquid chromatography–tandem mass spectrometry (LC–
490 MS/MS), limit of detection (LOD), limit of quantification (LOQ), Malawi Bureau of Standards
491 (MBS), multidrug resistance (MDR), oxidation–fermentation (OF), principal component analysis
492 (PCA), parts per billion (ppb), standard deviation (SD), trimethoprim-sulphamethoxazole (TMP-
493 SMX), too numerous to count (TNTC), Violet Red Bile Glucose agar (VRBG), and World Health
494 Organization (WHO).

495

496 **Ethics approval and consent to participate**

497 Ethical approval for this study was obtained from the Animal Health Committee (AHC) of the
498 Department of Animal Health and Livestock Development (DAHLD), Ministry of Agriculture,
499 Malawi (Approval No. DAHLD/AHC/01/2023/4). The study was conducted in accordance with the
500 Malawi Animal Welfare Guidelines (2019) and all conditions stipulated by the Animal Health
501 Committee. Permission to access the study sites and collect samples was obtained from the relevant
502 farm owners and authorities prior to sample collection. As this study involved animals only, informed
503 consent from human participants was not applicable.

504

505 **Consent for publication**

506 Not applicable.

507

508 **Availability of data and materials**

509 The datasets generated and/or analysed during the current study are available from the corresponding
510 author on reasonable request.

511

512 **Competing interests**

513 The authors declare that they have no competing interests.

514

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519

520 **Authors' contributions**

521 **TK:** Conceptualization, methodology, investigation, supervision, formal analysis, project
522 administration, writing – original draft, and writing – review & editing. **PM:** Formal analysis, data
523 curation, visualization, and writing – review & editing. **NM:** Investigation, field sample collection,
524 data curation, and writing – review & editing. **EC:** Laboratory analyses and writing – review &

525 editing. **PN:** Methodology, laboratory supervision, validation, and writing – review & editing. **RM:**
526 Methodology, laboratory supervision, validation, and writing – review & editing. **SM:** Interpretation
527 of findings and writing – review & editing. All authors read and approved the final manuscript.

528

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