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Antimicrobial resistance in dairy slurry tanks: a critical point for measurement and control

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Abstract

Waste from dairy production is one of the world's largest sources of contamination from antimicrobial resistant bacteria (ARB) and genes (ARGs). However, studies to date do not provide necessary evidence to inform antimicrobial resistance (AMR) countermeasures. We undertook a detailed, interdisciplinary, longitudinal analysis of dairy slurry waste. The slurry contained a population of ARB and ARGs, with resistances to current, historical and never-used on-farm antibiotics; resistances were associated with Gram-negative and Gram-positive bacteria and mobile elements (IS*Ecp1*, Tn*916*, Tn*21*-family transposons). Modelling and experimental work suggested that these populations are in dynamic equilibrium, with microbial death balanced by fresh input. Consequently, storing slurry without further waste input for at least 60 days was predicted to reduce ARB spread onto land, with >99% reduction in cephalosporin resistant *Escherichia coli*. The model also indicated that for farms with low antibiotic use, further reductions are unlikely to reduce AMR further. We conclude that the slurry tank is a critical point for prevalence and control of AMR, and that measures to limit the spread of AMR from dairy waste should combine responsible antibiotic use, including low total quantity, avoidance of human critical antibiotics, and choosing antibiotics with shorter half-lives, coupled with appropriate slurry storage.

Introduction

Antibiotics provided to food-producing animals account for 73% of global antibiotic sales (1), prompting concerns about the selection of antibiotic resistance bacteria (ARB) and genes (ARGs), and their migration from livestock and their environment to humans. ARB and ARGs associated with livestock can enter humans through consumption of animal products, e.g. contaminated meat (2, 3) and dairy (4, 5), or more indirectly, e.g. through land-application of animal waste, which may subsequently infiltrate crops (6, 7) and connected water resources (8, 9).

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Cattle production comprises 50% of global Livestock Standard Units (10), so has considerable environmental impacts that need to be mitigated (11). There are approximately 265 million dairy cows globally, producing high volumes of waste manure, estimated at 3 billion tonnes per year (www.faostat.org). In the UK, the site of this study, dairy farms are estimated to account for 80% (67 million tonnes) of total annual livestock manure production (12), with more cattle waste material applied to soil in England and Wales than swine and poultry combined (13).

Antibiotics are routinely administered to dairy cattle for treatment, and, in some cases, prevention of common illnesses, including mastitis and respiratory disease (14-16). Lameness, the most costly disease to UK dairy farms (17), is often prevented with application of antimicrobial metals (copper, zinc) or other chemicals (formalin, glutaraldehyde) in the form of footbaths (18), known to co-select for antibiotic resistance (19, 20). Dairy waste can therefore contain selective and co-selective pressures in the form of mixtures of antibiotics and assorted antimicrobials, as well as ARB, including Extended Spectrum Cephalosporin-Resistant (ESC-R) *E. coli* (21, 22), and genetic resistance determinants (23, 24). Thus, dairy waste may represent one of the world's most substantial routes for AMR to enter the environment, including onto fields and grasslands used for food production and into water ways.

To limit the risks of AMR, many countries have introduced responsible use policies, including reducing overall agricultural use of antibiotics (25), or of human critical antibiotics, including 3rd/4th generation cephalosporins (26). However, antibiotics and other antimicrobials remain necessary for safeguarding animal health and welfare. Thus, other countermeasures are also needed to reduce the transmission or prevalence of ARB and ARGs from dairy waste into the environment. For example, current UK guidelines suggest that storage of solid manure and slurry without fresh input for three months can ameliorate AMR risk (27), but no evidence is provided. Slurry storage is essential in the UK and other countries where dairy cows are housed indoors for large parts of the year,

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and where slurry cannot be spread onto land that is frozen or deemed nitrate vulnerable. Two European studies have assessed storage effects on dairy manure, finding that certain ARGs increased during storage (28, 29); however, this 'stored' effluent regularly received fresh input. Contrastingly, a survey of several US dairy farms evaluating a different set of ARGs did not detect clear storage effects on ARG abundancesHurst, Oliver (30).

Other dairy waste studies took a 'snapshot in time' (31-34), which does not allow for assessment of temporal stability of the resistome and the influence of storage. Factors such as temperature also influence the prevalence of enteric pathogens, indicator organisms and resistance phenotypes during manure storage (35-39). Meanwhile, studies assessing how cattle faecal resistomes respond to contrasting antibiotic management practices generally place emphasis on individual cattle (40-42), with different microbiomes, rather than the collective faecal output of the herd. Liquid-solid separation of manure may also influence the persistence of AMR (43).Therefore, there is a need for detailed longitudinal studies of AMR in dairy slurry and potential mitigations.

This study assessed three key questions about AMR in slurry and its relationship to antibiotic use and slurry storage: (1) does the slurry tank select for or against AMR; (2) how does the resistance content of the slurry tank relate to altered patterns of farm antibiotic use; and (3) can slurry storage help reduce AMR in slurry before application to land? Our interdisciplinary approach combined phenotypic, genomic, and metagenomic microbiological analyses with chemical analyses, antibiotic use records and predictive mathematical models, to provide a temporal evaluation of slurry tank content over six months. This was supplemented by concurrent mini-slurry tank experiments which facilitated the controlled study of isolated slurry. We designed the mathematical model to enable us to study the impact of farm practices that would be impractical or unethical to perform through purely empirical approaches. These included major changes to farm slurry handling, antibiotic reduction to a level that would threaten animal welfare, or the

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reintroduction of use of human critical 3rd or 4th generation cephalosporins. Thus, this study enables the identification of approaches to reduce the spread of AMR into the environment from an important source of such contamination.

Methods

Sample site

We surveyed a mid-sized, high performance commercial dairy farm in England, housing ~200 milking Holstein Friesian cattle at the time of study. Practice at this farm is typical of management methods at high-performance dairy farms, although all farms vary. Milking cattle are housed indoors on concrete, and all excreta are regularly removed from cattle yards by automatic scrapers into a drainage system terminating at the 3000m^3 slurry tank. The drainage system also receives used cleaning materials and wash water, used footbath containing zinc and copper, waste milk from cows treated with antibiotics, and rainwater runoff. An automated screw press (Bauer S655 slurry separator with sieve size 0.75 mm; Bauer GmbH, Voitsberg, Austria) performs liquid-solid separation prior to the slurry tank. Liquids enter the slurry tank semi-continuously, while solids are removed to a muck heap. Calves, dry cows, and heifers are housed separately from the milking cows. Faeces and urine from calves drain into the common drainage system, whilst dirty straw from calf housing is taken directly to the muck heap. Excess slurry can be pumped to an 8000m^3 lagoon for long term storage. Slurry from either the slurry tank or lagoon is used to fertilise grassland and arable fields.

Microbiological sampling, strain isolation, antimicrobial susceptibility testing and whole genome sequencing

Liquid samples were collected from the slurry tank on 17 dates between May and November 2017 (Table S1). *Escherichia coli* strains were isolated using Tryptone Bile X-

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Glucuronide (TBX) or MacConkey agar or TBX/MacConkey supplemented with 16 µg ml^{-1} ampicillin (AMP), or 2 µg ml^{-1} cefotaxime (CTX); or on CHROMagar ESBL™ agar. Putative *E. coli* isolates were subcultured onto TBX agar or TBX agar supplemented with 2 µg ml^{-1} CTX. *E. coli* strains were confirmed using oxidase (22) and catalase tests. Antimicrobial susceptibility testing (AST) using a range of antibiotic discs (Table S2) was carried out on 811 *E. coli* isolates in accordance with CLSI (44) guidelines. ESC-R *E. coli* strains were identified by phenotypic resistance profile as putatively *ampC* or CTX type, and confirmed by PCR (22). Presence of Tn*21*-like mercury resistance transposons within the *E. coli* isolates was initially screened for by growing isolates on LB agar containing 25 µg ml^{-1} $HgCl_2$. Their presence was confirmed by PCR (45). Genome assembly of selected ESC-R and mercury resistant *E. coli* strains using PacBio, was carried out by the Centre for Genomic Research (CGR), University of Liverpool, with methods for library preparation and sequencing as previously described (46) or by Illumina short read WGS by MicrobesNG (University of Birmingham, UK). Genome sequence analysis and annotation was conducted using Prokka (47), CSARweb (48), Snapgene viewer (Insightful Science; snapgene.com), Res Finder (49) and Plasmid Finder (50). Genome sequences are deposited with NCBI under BioProject PRJNA736866.

Metagenomics Sample collection and DNA extraction

Main tank Sample Collection

Samples were collected from the slurry tank monthly between June and October 2017, using a clean stainless steel bucket, and aliquoted into 2 large glass bottles with external PE protection. Three replicate extractions were performed on 250 µl of each sample using a PowerFecal Kit (Qiagen), according to manufacturer's instructions (15 extractions in total). DNA was quantified using a Qubit fluorometer (Invitrogen) while quality was assessed via Nanodrop 1000 (ThermoFisher). Extracted DNA was stored at 4°C pending sequencing.

Mini-Tank Experiments

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Miniaturised experimental slurry tanks were set up to assess the impact of storing slurry (control tanks) and to measure antibiotic stability. Twelve mini-tanks were situated on the farm from 23/4/2018 to 15/6/2018 at ambient temperature (mean 24 hour temperature in liquid ranged between 7° to 17°), protected from rain and direct sunlight, and containing 10L grab samples of slurry from the surface of the main slurry tank. Six different conditions were tested in duplicate (all amounts per litre): control; + SSD (0.2mL of slurry solids homogenised by stomacher, including 67 CFU of CTX-resistant *E. coli*); + SSD + 3μg cefquinome weekly addition; + SSD + 40μg cefalexin weekly addition; + SSD + 16.8g of footbath mix (Cu + Zn); + SSD + footbath + cefquinome). Mini-tanks were sampled four times (0, 2, 4 and 7 weeks after initial filling). Experimental conditions were mainly used for model calibration (Supplementary Text 1). *E. coli* were isolated and cultured as described above except MacConkey agar was not used. DNA was extracted and processed for sequencing as above. Antibiotic concentrations were measured as described previously Baena-Nogueras, Ortori (51) with further methods described in Supplementary Text 3.

Metagenomic Sequencing, Assembly and Analysis

Metagenomic sequencing of DNA extracted from the main slurry tank was performed by Liverpool Genomics using the Illumina HiSeq platform, and from the mini-slurry tanks by Edinburgh Genomics using the Illumina NovaSeq platform (150 bp paired end libraries in both cases). For the main tank, reads were trimmed with Cutadapt v1.2.1 (52) and Sickle v1.2.0.0 (53), while mini-tank reads were trimmed with Fastp v0.19.07 (54). Assembly was performed on trimmed reads using Megahit v1.1.3 (55). Main tank technical replicates were pooled by date and assembled using the settings: k-step 10; k-range 27-87. Mini-tank metagenomes were assembled individually (k-step ~20, k-range: 21-99). Metagenome sequences are deposited with the ENA under Study Accession PRJEB38990.

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Read-based searches for ARGs were performed with DeepARG v2 (56). ARGs were also identified on contigs (>1.5 kb length) in order to investigate the wider genetic context of the core resistome using ABRicate v1.0.1 (57), using MegaRes 2.0 for ARGs and metal resistance genes (MRGs) (58) (including experimentally verified MRGs; genes requiring SNP validation were excluded) and ACLAME 0.4 for MGEs (59). All data were analysed with stringencies: >60% gene coverage, >80% identity(60). Lastly, the BacMet2 database (61) was screened against translated peptides (based on Prodigal (62) output) from meta-assemblies of the main and mini-tanks (stringencies: >60% sequence identity and match length >50% of peptide length).

Taxonomic assignment of reads was performed using Kaiju v1.6.2 (63), with default settings. The reference database used was a microbial subset of the NCBI database (64), including additional fungal and other microbial eukaryotic peptide sequences. Contigs of interest were assigned putative identities using NCBI-nucleotide BLAST (65) (MegaBlast(66), highly similar sequences).

For both ARG and taxonomic assignments, statistical comparisons were carried out using the DirtyGenes likelihood ratio test (67), using randomized resampling (n=1000) from the null distribution to establish p-values.

Water Quality Analysis

Water quality analysis was performed on the same samples as for microbiological culturing. For each sample, 2.5L was initially sampled. Probes were used to assess the pH (Hach PHC201), dissolved oxygen (Hach LDO101) and NaCl (Hach). The probe tip was rinsed in Milli-Q water (Merck), dabbed dry and submerged into the bottle containing slurry and left to equilibrate. The sample was then homogenized by shaking vigorously before decanting into a 250mL bottle for analysis using a Hach DR3900 Laboratory Spectrophotometer with cuvette test kits: sulphate (LCK153); ammonium (LCK303); chloride (LCK311); copper (LCK329); LATON total nitrogen (LCK338); nitrate (LCK340); nitrite (LCK342); phosphate (LCK348); zinc (LCK360); COD (LCK514); and TOC (LCK381). Standard procedures are available from https://uk.hach.com.

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Mathematical Model

A mechanistic, multi-strain model of AMR in the slurry tank was constructed to simulate a range of relevant farm management scenarios that would have been impractical or unethical to carry out empirically. In brief, it is a coupled ordinary differential equation model of bacterial populations including logistic growth, death (baseline and antimicrobial induced), horizontal transfer and fitness cost of resistance, inflow and outflow (68, 69). The model considered mobile resistance to penicillin, tetracycline, cephalexin, cefquinome, copper, and zinc, and was simulated for a full year in order to capture the recorded input of cephalexin and other antibiotics. The choice of resistances reflects our interests in ESC-R *E. coli* strains, and the risk of environmental contamination by mobile genes following slurry spreading. Full model description is provided in Supplementary Text 1, equations in Supplementary Text 2 and parameter values in Table S4. This model was deposited in BioModels (70) as MODEL1909100001. The secondary storage model is derived from this model by duplicating equations for each storage vessel (70) and also deposited as MODEL1909120002. A reduced model was used for parameter inference from mini-tank data. Model simulations were carried out in Matlab using the ode45 solver.

Results

Resistance to antibiotics with historic, current and no documented farm use

The majority of antibiotics administered to milking cows during the sampling period were aminocoumarins, aminoglycosides and beta-lactams delivered in combination, and beta-lactams and tetracyclines administered individually (Table S3). The last recorded use of sulphonamides (sulfadoxine) was in June 2016; of first generation cephalosporins (cephalexin) was in April 2017 (shortly before the start of the sampling period); of third generation cephalosporins (ceftiofur) was in January 2016; and of fourth generation cephalosporins (cefquinome) was in August 2015. Residual antibiotics or ARB associated with historical use could potentially be present in sludge at the bottom of the tank that

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cannot be piped for spreading. Smaller quantities of antibiotics are also given to youngstock; their waste does not enter the slurry system.

The dominant resistance phenotypes of cultured *E. coli* isolates from the slurry tank (Figure 1a) were ampicillin (34.6%), cefpodoxime (39.3%), cefotaxime (29.6%) and streptomycin (26.5%); other common phenotypes included tetracycline (13.6%), chloramphenicol (10.7%) and nalidixic acid (9.6%). Multidrug resistant *E. coli* strains (≥3 different antibiotic classes, Magiorakos, Srinivasan (71)) represented 37% of the cultured isolates (Figure 1b), detected in strains isolated on both antibiotic-supplemented and non-supplemented media. Of these isolates, 12 cefotaxime resistant *E. coli* strains were sequenced to characterize the resistance genes and mobile elements carrying them. Three carried IS*Ecp1* CTX-M-15, additionally carrying *qnrS* and *tetM* within the *ISEcp1* element. The other sequenced ESC-R strains were chromosomal *ampC* mutants.

In main slurry tank metagenomes, eight resistance classes account for 98% of the ARGs identified in reads (Figure 1c): multidrug resistance genes (36.7%); tetracycline resistance genes (21.6%); macrolide-lincosamide-streptogramin (MLS) resistance genes (21.4%); aminoglycosides (7.3%); beta lactams (4.5%); peptides (4.0%); bacitracins (1.6%) and glycopeptides (1.2%). MRGs were also identified (*mer*: mercury; *cop*, *cus*, *pco/sil*: copper, copper/silver; *cad*, *czc:* cadmium*,* cadmium/zinc/cobalt; *ars*, arsenic/antimony; *pbr* lead resistance). In equivalent metagenome read assemblies, MLS and tetracycline ARGs were most frequently detected (70 and 46 contigs, respectively). Few MRGs were detected in main tank metagenome assemblies, limited to TCR copper resistance genes (5 contigs).

Overall, the identification of aminoglycoside, beta-lactam (excepting 3rd/4th generation cephalosporins) and tetracycline resistance genes and phenotypes reflect current or recent farm antibiotic use, while the presence of zinc and copper resistance genes reflect transition metal use. The presence of sulphonamide and cephalosporin resistance genes and phenotypes may be due to historical use, or reflect widespread environmental

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298 occurrence (72). The prevalence of MLS resistance genes is unlikely to be associated
299 with antibiotic use, as there is no recorded MLS use for milking cows.
300
301 **Slurry tank properties and AMR remained stable due to frequent inputs**
302 Water quality measures were largely stable (Figure 2a), with some fluctuations in July
303 and August likely to be associated with mixing of slurry in the tank prior to spreading on
304 fields. The relative contribution of the dominant drug-resistance categories listed above
305 remained unchanged throughout the sampling period (Figure 1c; p=0.172, DirtyGenes
306 test). Likewise, taxonomic analyses of read data showed the time-stable dominance of
307 six bacterial phyla with at least 1% prevalence (Figure 2b; p=0.254, DirtyGenes test):
308 Bacteroidetes (13.8%), Firmicutes (13.7%), Proteobacteria (4.7%), Spirochaetes
309 (2.9%), Euryarcheaota (1.9%) and Tenericutes (1.4%). These phyla only account for
310 38% of the microbial community: there is considerable diversity in the tank with 178
311 phyla identified (Table S4).
312 The overall numbers of *E. coli* identified through culture-based enumeration also
313 remained stable (Figure 2c), with concentrations of 4.23±0.40 (Log_{10} CFU mL^{-1}) on TBX
314 plates and 4.29±0.46 (Log_{10} CFU mL^{-1}) on MacConkey media. *E. coli* strains resistant to
315 ampicillin (TBX/Amp 16 µg mL^{-1}) were stable at concentrations of 3.99±0.43 (Log_{10} CFU
316 mL^{-1}), i.e. ~58% of cultured *E. coli* strains. *E. coli* that could be cultured on cefotaxime
317 selective plates (TBX/CTX 2 µg L^{-1}) were detected on only five of 17 sampling dates, with
318 counts below 10 colonies per plate on all but one day (22nd August). Thus, cefotaxime
319 resistant *E. coli* were present at low levels, but could not be reliably quantified. The full
320 AST profiles of the 811 isolates also show consistency over time, with some random
321 variation, both on antibiotic-free and antibiotic-supplemented media (Figure 3).
322
323 **Model predictions are consistent with microbial data**
324 In the mathematical model, predicted resistance to penicillins fluctuated between 0.4%
325 and 6.4% and cephalosporins between 0.5% and 7.9% (Figure 4a), i.e. both present but
326 low, despite frequent inflow of antibiotics into the tank (Figure 4b). Resistance to

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tetracycline increased from low initial levels to fluctuate around ~25% of the *E. coli* population (Figure 4a), before slowly declining over the longer term, reflecting the decline in tetracycline use later in the year. These predicted levels of tetracycline and cephalosporin resistances are concordant with the empirical phenotype above. Penicillin resistance in the model is lower than observed empirically, probably because resistance in the model is plasmid-borne, while many strains have chromosomal mutations of *ampC* or chromosomally located resistance genes that could be mobilised (e.g. IS*Ecp1*CTX-M-15 elements). The model predicts that zinc resistance is highly prevalent, rising to fluctuate around 80%, with co-occurrence of tetracycline and zinc resistance, typically fluctuating between 10 and 15%, consistent with predictions that the metal concentrations in the tank are co-selective (69).

Associations of ARGs with other ARGs, integrons and Gram-positive taxa

Several metagenome contigs contained two or more ARGs, MRGs or associations with MGE markers in both the main tank (37 contigs) and mini-tank metagenome assemblies (101 contigs) (Figures S1 and S2). These include ARGs belonging to the same resistance gene group, e.g. *aph*3 and *aph*6 (both aminoglycoside resistance genes; Figure S3a) which were co-localised on five main-tank and eight mini-tank contigs; as well as genes associated with entirely different antibiotic resistance classes, e.g. *ant*6 and *tet*44 (aminoglycoside and tetracycline resistance, respectively; Figure S3b) were co-localised on two main-tank and eight mini-tank contigs. In other mini-tank contigs, *aph*3-*aph*6 were additionally co-resident with either a sulphonamide (*sul*2, 1 contig) or tetracycline (*tetY*, 1 contig) resistance gene. *tetM* was embedded within the widely documented Tn*916* transposon (18 *tetM* contigs in total, nine of which were linked with Tn*916* elements). The two largest Tn*916*-like contigs (18.3-18.9 kb) appear to be carried within Gram-positive bacteria, possibly *Streptococcus* spp. or *Enterococcus* spp. (NCBI-BLAST, ~99.96% identity, ~91% query coverage; Figure S3c). Furthermore, 21.4% (n= 6/28) of main and mini-tank contigs containing *cfxA* (class-A beta-lactamase) were co-localised with mobile elements.

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Further identification of mobile resistance cassettes was through a screen of all *E. coli* strains for phenotypic mercury resistance as a marker for Tn*21* carriage. Sequence analysis of mercury resistant *E. coli* strains showed that three carried Tn*21* variants carrying the integron intI*2* conferring co-occurrence of combinations of penicillin, sulphonamide, aminoglycoside and quaternary ammonium compound resistances.

Waste management for AMR reduction

We investigated the use of slurry storage to ameliorate resistance through a combination of empirical and modelling work. In the mini-tanks, we found that storage of slurry without inflow rapidly decreased the total concentration of cultured *E. coli* cells (Figure S6a), as well as *Escherichia, Pseudomonas and Klebsiella* spp. sequences identified by metagenomics (Figure 5). Reads assigned to gut-associated anaerobes belonging to Bacteroidetes including *Bacteroides* spp., *Alistipes* spp. and *Prevotella* spp. declined in steps. In contrast, the relative abundance of *Acinetobacter* spp. gradually increased until week four, before declining again by the end of the experiment (Figure 5).

The prevalence of beta-lactam resistance genes declined considerably in <2 weeks (Figure 6a). The overall relative abundance of tetracycline resistance genes declined marginally over 7-weeks of storage (Figure 6b); however, different patterns were observed with different gene groups: *tetY* (Figure 6c) and *tet*40 (Figure 6d) declined sharply within two weeks, while others, e.g. *tetM* (Figure 6e) were maintained in stored slurry. According to BLAST analysis against the NCBI database, mini-tank contigs containing *tetY* (2 contigs) were likely associated with Gamma-Proteobacteria, while *tet40* (6 contigs) was consistently linked to Firmicutes. Similarly, *tetM* was typically associated with Firmicutes (7 of 16 contigs; >89% sequence coverage, >99% sequence identity), more specifically Bacilli. The proportion of MLS ARGs remained comparatively stable throughout (Figure 6f), consistent with their presence not connected with patterns of MLS use on the farm.

We implemented a two-stage in series storage mathematical model to consider whether the storage of slurry in the main tank, without fresh inputs, would reduce AMR in slurry prior to land application. The model predicted that after only four days of storage, 50%

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of the amoxicillin- and cefalexin-resistant *E. coli* are removed, and after 60 days of storage, only 0.29% of cefalexin-resistant and 0.00001% of amoxicillin resistant *E. coli* remained (Figure 7a). However, the model predicts that tetracycline resistant bacteria will increase over this time by 25% due to ongoing selective pressure and low fitness cost. Importantly, multidrug resistant *E. coli* become undetectable.

Simulations of altered antibiotic use support criteria for responsible use

Simulations of on-farm antibiotic use (~9.7 mg/Population Correction Unit (PCU) in 2017) result in low levels of penicillin and cephalosporin resistance, consistent with the empirical data. We simulated further reductions in antibiotics entering the tank to either 50% or 10% of current use. Neither reduction had a material impact on either resistance (Figure 7b) but there is a small reduction in tetracycline resistance (33% reduction in resistance at 10% usage) because of the reduced selective pressure for tetracycline resistance.

Very few cephalosporin resistant *E. coli* were detected in the farm samples (detailed above). Thus, we also simulated a return to use of the critically important 4th generation cephalosporin (cefquinome) in place of cefalexin (1st generation), assuming that cefquinome resistance also confers resistance to cefalexin. After accounting for the lower recommended dosage of cefquinome relative to cefalexin, we found cefquinome use increased resistance to both cefquinome and cefalexin of only 0.65% and 0.35% increase respectively (Figure 7c). To represent high antibiotic use following an outbreak of disease, we simulated 50 mg/PCU of cefquinome used in place of cefalexin. Such a scenario was predicted to select an increase of cefquinome resistance of only 3.55%.

Discussion

The slurry tank is a critical measurement and control point for AMR

The bacterial community and ARGs in the slurry tank appear to be maintained in a state of dynamic equilibrium, with a balance between input of fresh microorganisms from the

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cattle, and decline, as observed in the mini slurry-tank experiments. This equilibrium is also evident in the observed stability of the virome of the same tank over the same sampling period (73). The slurry tank maintains an array of ARGs, many of which have been found in other animal wastes. These include MLS genes such as *mefA* (24, 29, 74) and the *cfxA* group of beta-lactamase genes (24, 74, 75). The association of *cfxA* with Gram-positive organisms suggests that AMR phenotyping should routinely include a Gram-positive sentinel; *Enterococcus* spp. may be suitable because of their use in water quality analysis (76) and the inclusion of *E. faecium* in the ESKAPE pathogens list (77). Tetracycline resistance genes such as *tetW* and *tetM* have also been frequently found in cattle and swine waste (29, 78, 79). Although present in low quantities relative to other ARGs, *tetM* has the potential for selection and possible mobilisation (*e.g.*, IS*Ecp1* or Tn*916*-like elements). Consequently, the tank appears to be a critical sampling location, representative of the AMR status of the farm as a whole, reflecting current and previous antibiotic use. The presence of resistance genes to antibiotics with no recorded use (e.g. quinolone resistance, MLS genes) are likely to reflect broader environmental, and possibly human, input into the farm microbiome.

At a superficial level, the slurry tank appears to meet many criteria presumed to define a 'hotspot' for AMR, which cite a high abundance of bacterial populations and the routine presence of antimicrobial residue (80). However, the concept of an AMR 'hotspot', where bacterial and antimicrobial abundance are assumed to lead to increases in AMR prevalence, alongside the related concept of 'reservoir', assumed to represent the nascent AMR genes circulating in the environment poised to be mobilised through antimicrobial exposure, are open to critique (81). Our findings suggest that the tank, rather than generating resistance, can ameliorate resistance, depending on the waste management practice, and that slurry be stored for at least two months without fresh slurry inputs to the system/tank. Thus, the tank is neither a hotspot nor a reservoir, but, if managed appropriately, can be a critical control point for reducing the transmission of ARGs and ARB from livestock into the wider environment.

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Agricultural AMR policy should combine responsible antibiotic use with effective waste management

Policy and industry guidance to reduce AMR focus on reduced or responsible agricultural antimicrobial use (25, 82, 83), including the cessation of use of human critical antibiotics. Our findings provide evidence in support of responsible use. Simulations of reductions below the already low level of 9 mg/PCU did not predict reductions in penicillin and cephalosporin resistance below current levels. However, reduced tetracycline use led to reduced tetracycline resistance, associated with the environmental stability of this antibiotic, suggesting that prudent antibiotic use could also include antibiotic choice encouraging use for those with shorter half-lives where medically appropriate. While our findings suggested that use of 3rd and 4th generation cephalosporins did not lead to substantial increases associated resistances, once such resistances are established, relevant genes, e.g. CTX-M, can be selected for by 1st generation use. Although UK policy initiatives have greatly reduced the use of 3rd/4th generation cephalosporins on UK dairy farms, globally their use remains prevalent, e.g. Ceftiofur (3rd generation cephalosporin) is routinely used in the US to treat metritis (84, 85) and mastitis (86). Eliminating the use of these antibiotics in agricultural production should still be an important goal of national and global policies to mitigate the environmental dissemination of AMR (87).

A policy focus on antibiotic use is limited because of the need to use antibiotics to treat sick livestock. We also showed that waste management practice provides an additional mechanism to control AMR, by reducing the prevalence of resistance genes and key microbial phyla in slurry prior to soil amendment. Specifically, secondary storage of slurry for a period of 60 days, without fresh inflow, would significantly reduce the levels of ARB within the tank, representing an opportunity for rational farm design and practice to minimize AMR outcomes. This result is also concordant with other practices for mitigating AMR on farms, including the use of anaerobic digestion (79, 88), vermicomposting and solid-liquid separation (43).

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Two qPCR-based studies surveying Finnish swine and dairy farms reported that storage of animal manure slurry coincided with significant increases in select tetracycline, sulphonamide and aminoglycoside resistance genes when compared to fresh manure (28, 29). However, the farms involved in these studies used storage systems which received regular fresh inflow during the sampling period. Our metagenomic analyses of mini-tanks indicate that in the absence of fresh input a range of ARG classes decline (e.g. aminoglycoside and beta-lactam ARGs) or remain relatively stable (e.g. MLS ARGs). Moreover, culture-based results confirm an overall reduction in antibiotic resistant *E. coli* in slurry stored without inflow. Collectively, this provides empirical evidence supporting existing UK guidelines regarding the storage of slurry without further input as a means of reducing environmental exposure to AMR determinants.

Evaluation of co-selection needs alternative approaches

Aminoglycoside, tetracycline and sulphonamide resistance genes were found on the same contigs. The result is consistent with sulphonamide resistance being co-selected by concurrent use of multiple antimicrobials because aminoglycosides and tetracycline were the two antibiotic classes used most during the sampling period. We anticipated finding evidence of co-occurrence of ARGs and MRGs in assembled metagenomic data, in accordance with other studies (19, 24, 89). However, apart from antibiotic resistance associated with Tn*21*-like elements carrying integrons, we found no evidence for such linkage in the slurry metagenomes or sequenced *E. coli* strains. This lack of evidence might not be evidence of absence of ARG-MRG co-occurrence, as these genes may not necessarily be genetically linked on a chromosome or on plasmids, and yet still be subject to co-selection if they reside in the same cell. Accordingly, the use of long-read or hybrid genome sequencing of strains selected for zinc or copper resistance may be more appropriate for detecting the co-occurrence of ARGs and MRGs (90).

Conclusions

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We have conducted a longitudinal, interdisciplinary study of the dynamics of AMR in a dairy slurry tank. The microbiota was in a state of dynamic equilibrium, with fresh input of bacteria from the animals balanced by natural decay. Antibiotic resistance was maintained, reflecting current and previous veterinary practice, as well as interaction with the broader environment. The slurry tank is therefore both a natural measurement point for on-farm resistance, as well as a control countermeasure point for resistance being released into the wider environment (land and water). The spread of antibiotic resistance into the wider environment through slurry application can be mitigated by a combination of responsible antibiotic use, including low total quantity, avoidance of human critical antibiotics, and antibiotic choice with shorter half-lives, with slurry storage. These approaches can mitigate spread of AMR into the environment from one of the world's largest sources of AMR pollution.

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Michelle Baker: Methodology, Formal Analysis, Investigation, Writing – Original Draft, Visualization

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Richard Helliwell: Investigation, Writing – Original Draft

Elizabeth King: Methodology, Investigation, Supervision

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Henry Todman: Methodology, Investigation

Charlotte J. Gray-Hammerton: Investigation

Ryan Cook: Data Curation

Alexander C. W. Pritchard: Investigation

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David A Barrett: Conceptualization, Supervision, Acquisition of Funding

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Jan-Ulrich Kreft: Conceptualization, Acquisition of Funding, Supervision, Writing – Reviewing and Editing

Jon L Hobman: Conceptualization, Acquisition of Funding, Supervision, Writing – Reviewing and Editing

Dov J Stekel: Conceptualization, Formal Analysis, Supervision, Writing – Reviewing and Editing, Project Administration, Acquisition of Funding

Data Availability

Genome sequences are deposited with NCBI under BioProject PRJNA736866. Metagenome sequences are deposited with the ENA under Study Accession PRJEB38990. Mathematical models are deposited in BioModels as MODEL1909100001 and MODEL1909120002. All other data, including all details of accession numbers of genome and metagenome sequences, are on Figshare (https://figshare.com) under project number 133176.

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849 Figure Legends

850 Figure 1: **Antimicrobial resistance phenotypes and reads in the slurry tank** (a)
851 Resistances to a panel of 16 antibiotics (Supplementary Table 2) largely do not depend
852 on the type of supplemented media used. (b) The number of resistances per isolates;
853 37% of cultured isolates resistant to three or more antibiotic classes. These resistances
854 are seen on all types of media. (c) Proportion of ARGs mapped to different antibiotic
855 resistance classes (% reads). The metagenomic resistance profile is largely stable over

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time. There appears to be a gradual increase in the proportion of aminoglycoside and beta lactam resistance genes, which could be seen as consistent with antibiotic use during that period, but there is no statistical significance to the changes in proportions. ARGs are also reasonably consistent with observed phenotype data.

Figure 2: **Stability of microbial ecosystem, *E. coli* counts and water quality measures**. (a) water quality analysis from samples taken from the slurry tank over a five month period concurrent with microbial counts. Water quality measures are generally stable, with some fluctuations concordant with slurry use. (b) Six taxonomic groups accounting for at least 1% each of microbial reads show stable abundance in time. There is considerable diversity; these groups only account for 38% of reads, with all reads mapped to 178 different microbial phyla. (c) Counts of *E. coli* concentrations showing *E. coli* on TBX and MaConkey plates (all *E. coli*), TBX and AMP plates (*E. coli* resistant to ampicillin) and on CTX plates (ESC-R *E. coli*). Overall *E. coli* abundance is stable throughout the sampling period, as are counts of ampicillin resistant strains. CTX-resistant *E. coli* are only observed on five sampling days, and on all of those occasions at levels too low to be reliably quantified. The data for the other days are below the limit of detection of the method used and are plotted at 0 for ease of display.

Figure 3: **Antibiotic susceptibility testing of *E. coli* isolates shows diverse but stable range of phenotypic resistances.** In each panel, the heatmap shows the proportion of strains resistant to each of 16 different antibiotics on each of the sampling dates. Grey bars indicate no use of those plate types on those dates. (a) plates without antibiotic supplement; (b) plates supplemented with ampicillin; (c) plates supplemented with cephalosporins. In all cases, the patterns of resistances are stable in time. Cephalosporin supplemented plates identify more resistant strains than other plates, including to other antibiotic classes, including tetracyclines and quinolones.

Figure 4: **Model simulations of antimicrobials and antimicrobial resistance in the slurry tank.** (a) Model prediction of resistant *E. coli* populations in the slurry tank over a

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885 year's period, given (b) antibiotic usage on farm in 2017. Resistance groups are not
886 mutually exclusive. The resistances are reasonably stable once the model simulation
887 reaches its steady state, with fluctuations resulting from periodic removal of slurry for
888 use as fertilizer. (b) Mass (in mg) of oxytetracycline, cefalexin and amoxicillin given
889 during 2017 together with model simulation predicting concentrations (in mg L^{-1}) of
890 these antibiotics in the slurry tank over the same period. Observe that tetracycline is
891 present in the tank, despite intermittent use, due to its high environmental stability. This
892 explains the consistent proportion of tetracycline resistance. The two beta lactam
893 antibiotics decay more rapidly after use.
894

895 Figure 5: **Storage without further waste-addition leads to a decline in select**
896 **bacteria.** Relative abundances of *Escherichia* spp., *Pseudomonas* spp., *Klebsiella* spp.,
897 *Bacteroides* spp., *Prevotella* spp. and *Alistipes* spp. in stored slurry based on
898 metagenomic short-read data. *Escherichia* reads from metagenomics are concordant
899 with culturing data (viable *E. coli* counts in CFU/ml over time are given in Figure S6a),
900 both showing a stepwise decline. *Pseudomonas* and *Klebsiella* also show a stepwise
901 decline. *Bacteroides*, *Prevotella*, and *Alistipes* show a gradual decline. *Acinetobacter*
902 increase over the first four weeks before declining.
903

904 Figure 6: **Impact of mini tank storage on selected ARGs based on DeepARG**
905 **analysis.** Relative abundance (percentage of reads) of (a) beta lactam ARGs; (b)
906 tetracycline ARGs; (c) *tetY*; (d) *tet40*; (e) *tetM*; (f) MLS ARGs. The decline in beta
907 lactam reads is consistent with other data. Tetracycline ARGs show different patterns for
908 different genes. The persistence of MLS ARGs is consistent with their presence not
909 related to lack of MLS use on the farm.
910

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911 Figure 7: **Model simulations of altered farm practise or antibiotic use** (a) Storing
912 slurry without fresh inflow for 60 days is predicted to reduce resistance. Cephalexin
913 resistance is reduced by more than 99.99% while amoxicillin resistance is reduced by
914 more than 99%. (b) Model predictions of current antibiotic usage (9.7 mg/PCU)
915 compared to a 50% reduction (4.85mg/PCU) and 90% reduction (0.97mg/PCU) show
916 negligible impact on slurry tank resistance levels. (c) Model predictions of the change in
917 resistant *E. coli* in the tank when using a 4th generation cephalosporin instead of a 1st
918 generation cephalosporin on low, medium and high antibiotic usage farms showing
919 increased resistance to all relevant antibiotics.
920
921

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Figure 1

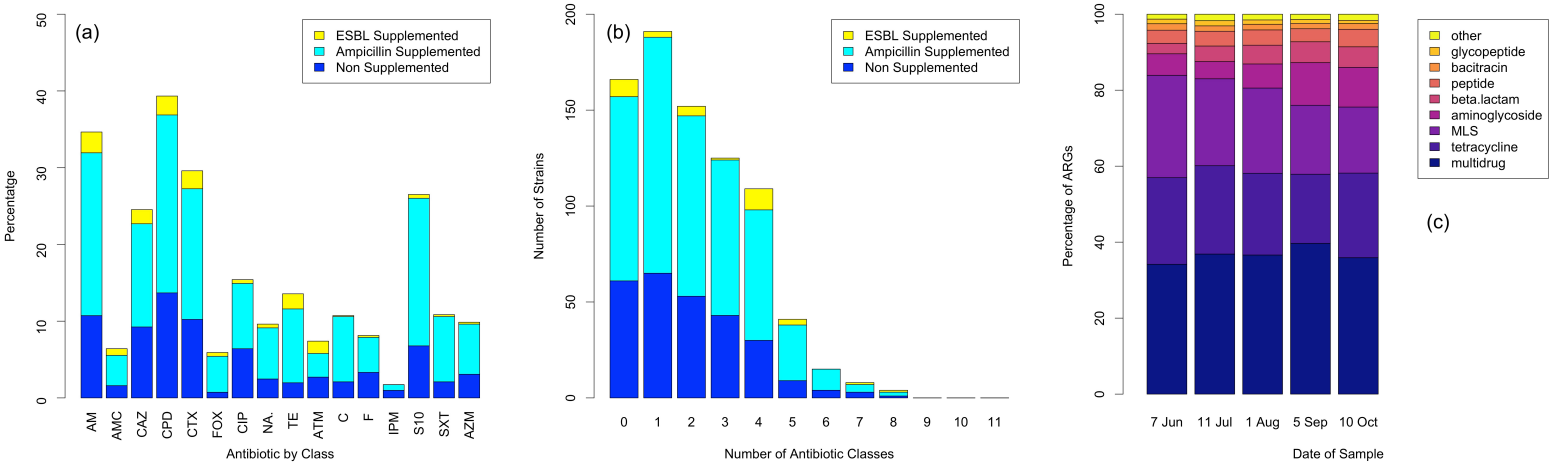

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Figure 2

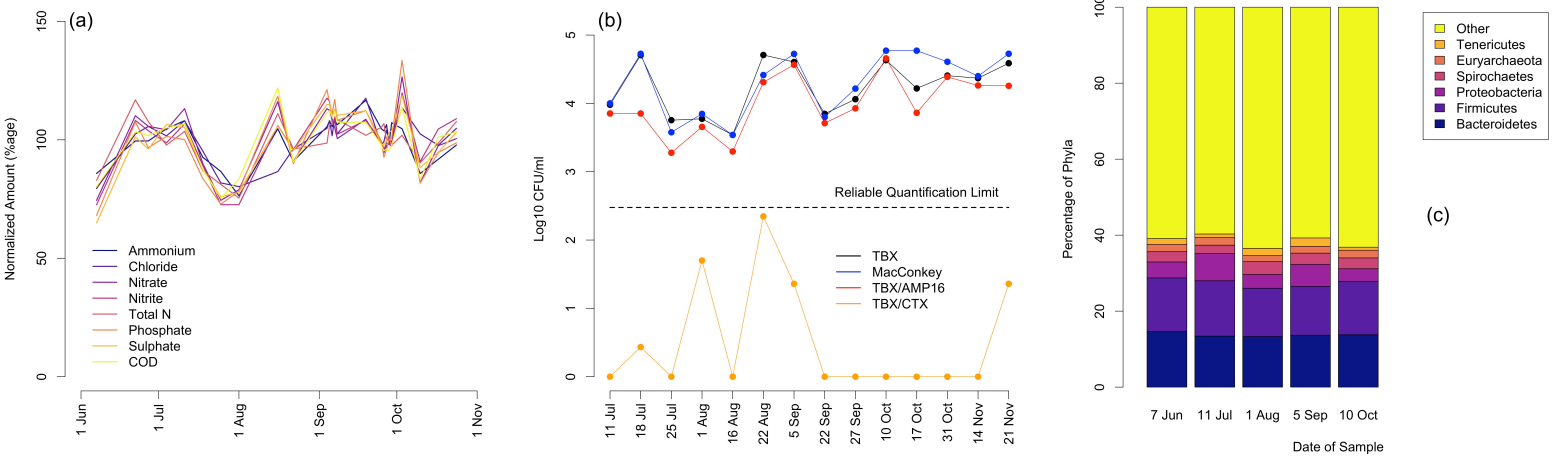

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Figure 3

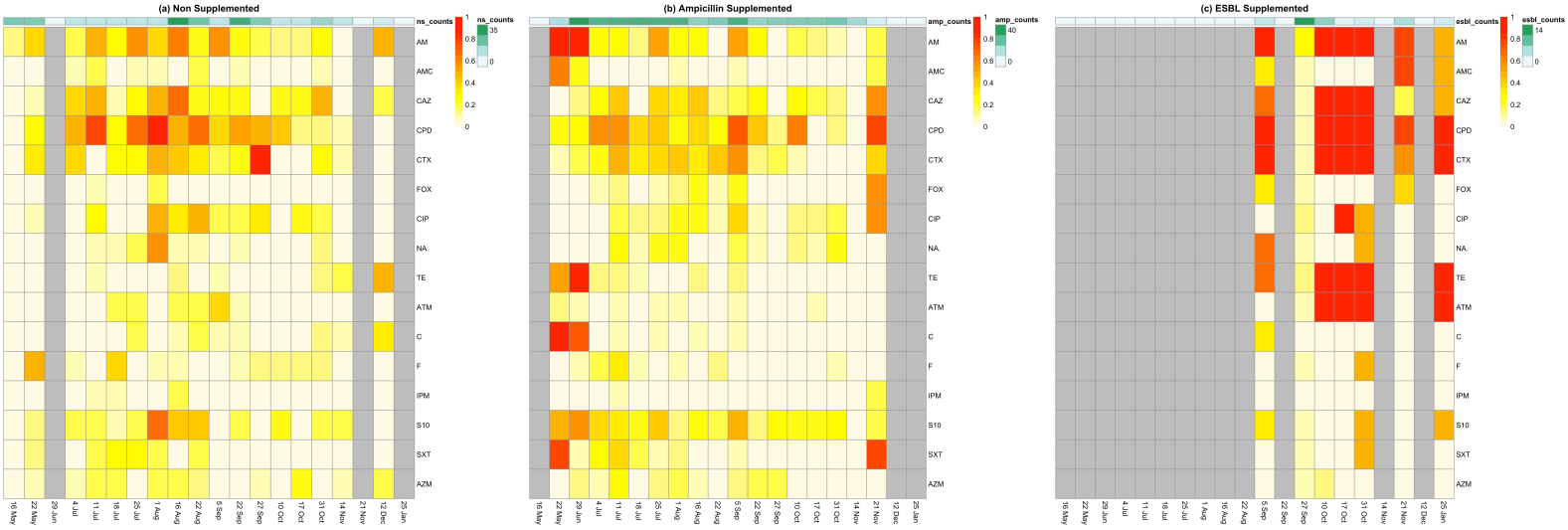

Figure 4

bioRxiv preprint doi: https://doi.org/10.1101/2022.02.22.481441; this version posted February 22, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

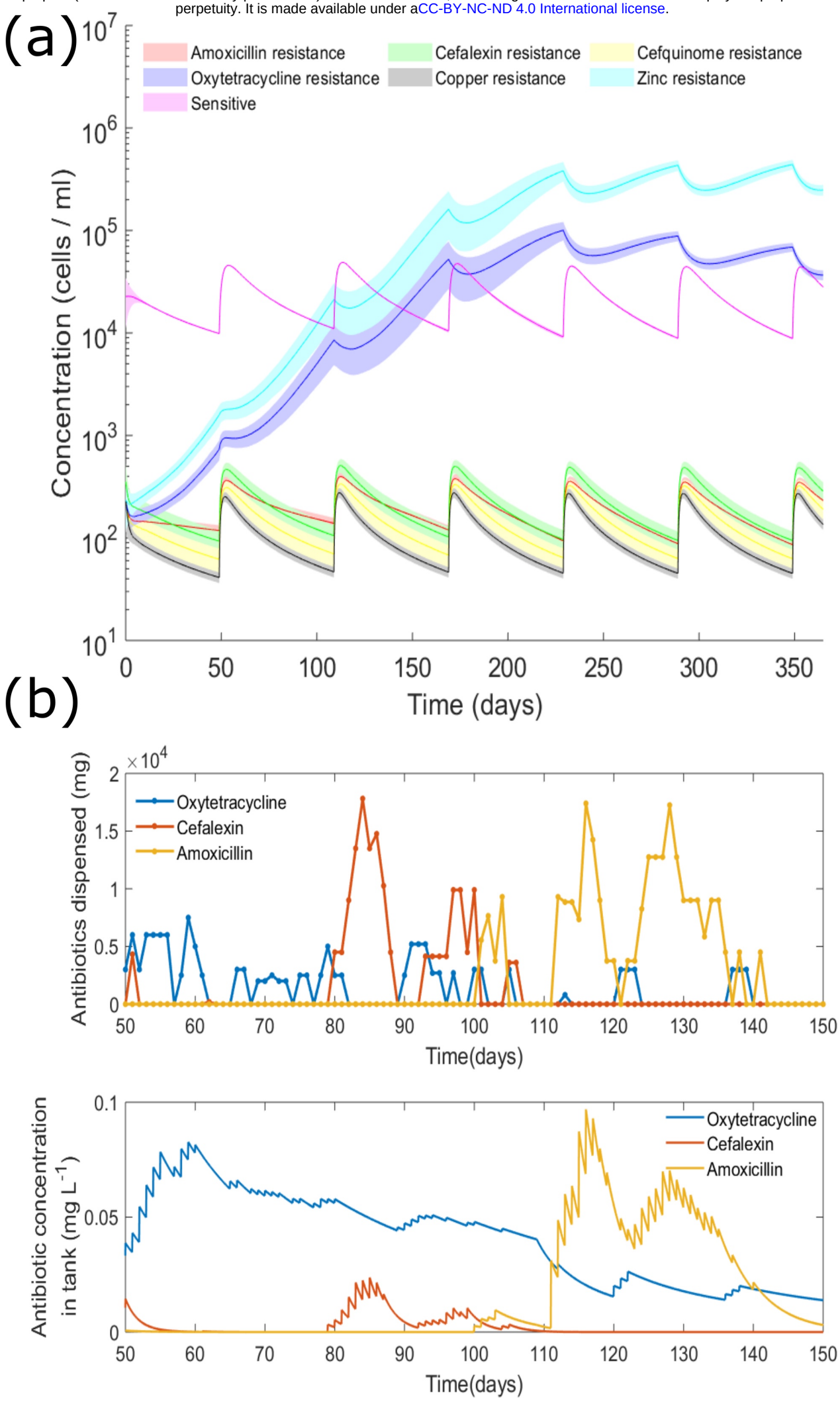

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Figure 5

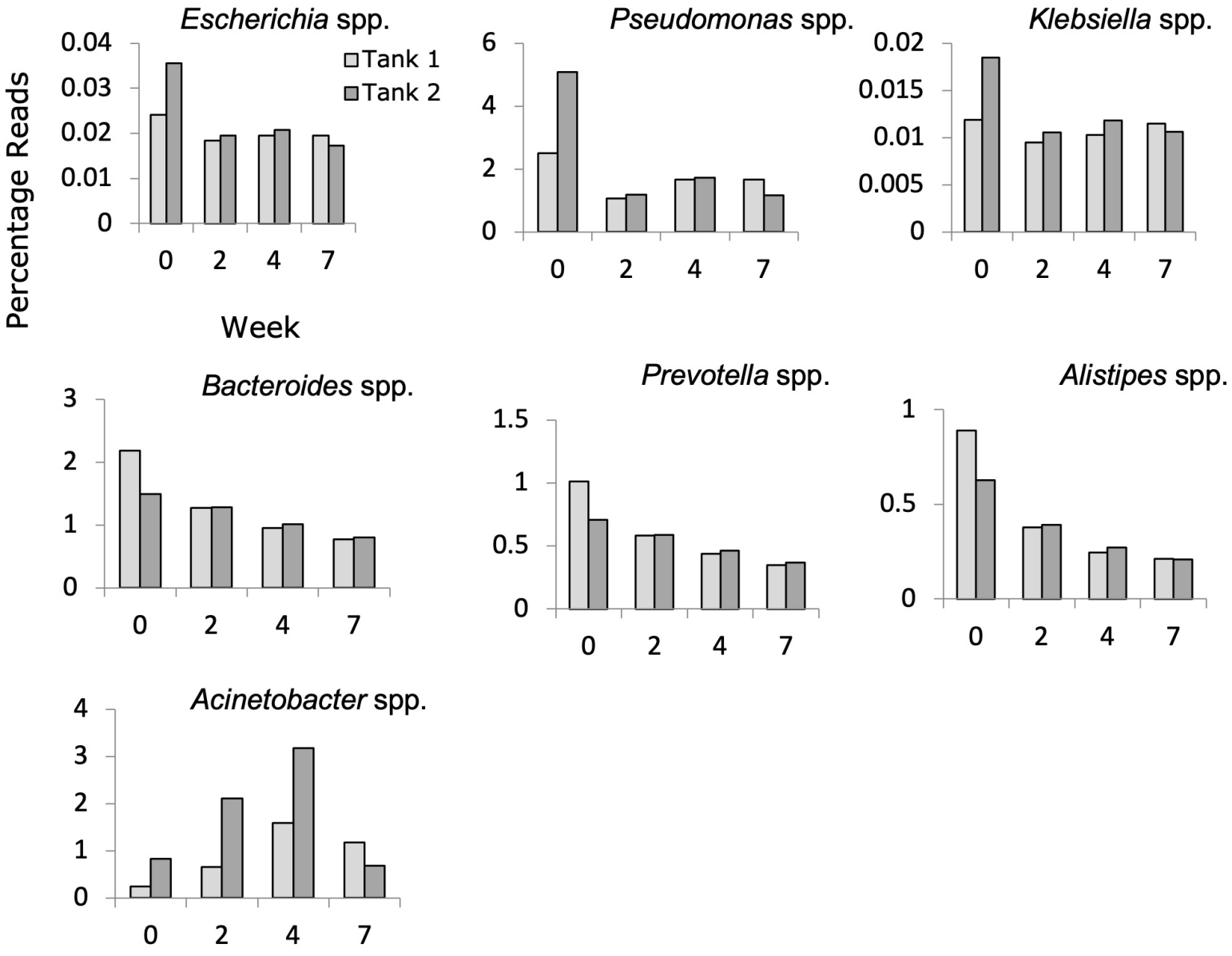

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Figure 6

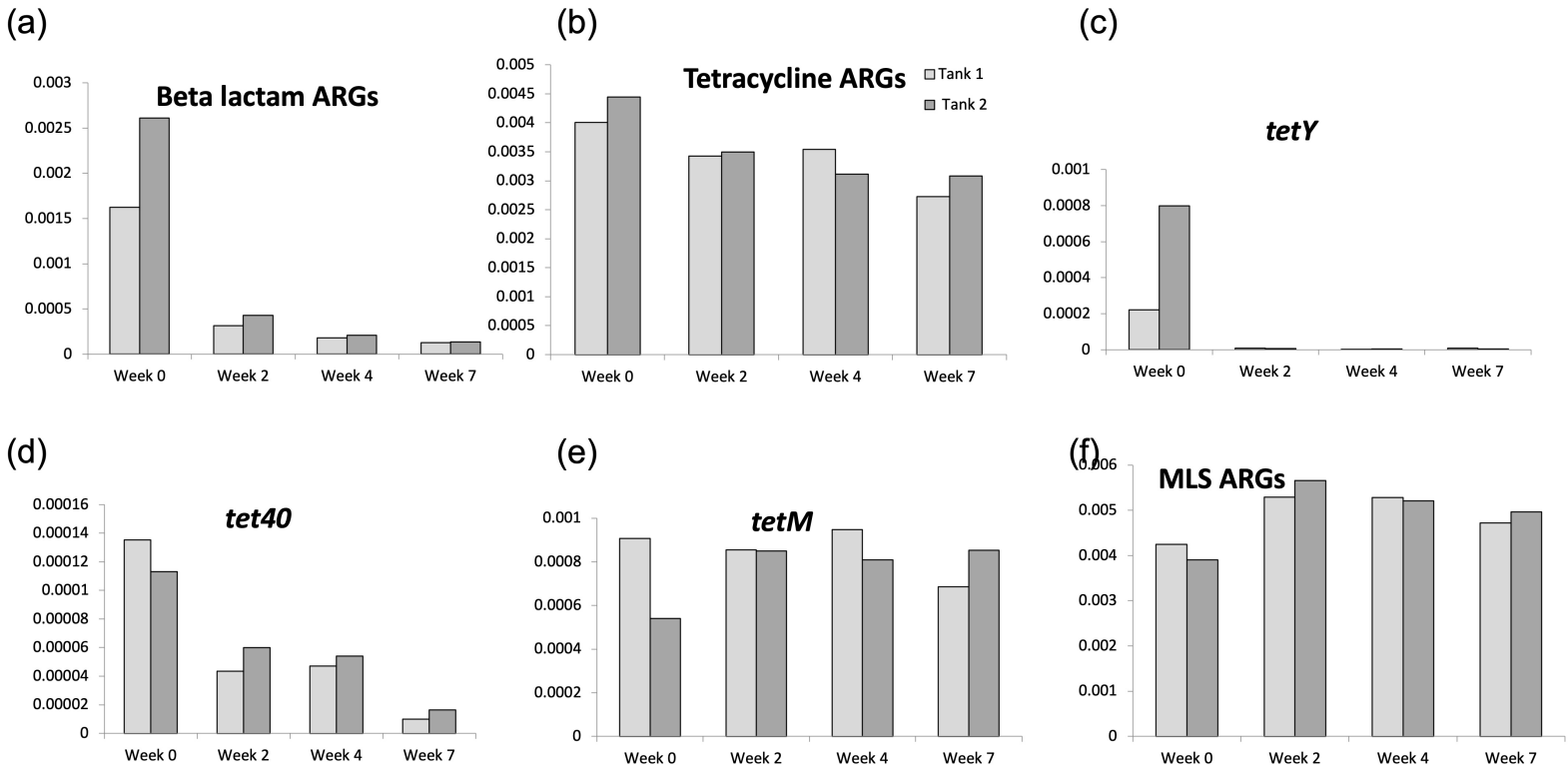

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Figure 7