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Identifying associations between management practices and antimicrobial resistances of sentinel bacteria recovered from bulk tank milk on dairy farms

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ABSTRACT

There is increasing emphasis on the need to reduce antimicrobial use (AMU) on dairy farms to reduce the emergence of resistant bacteria which could compromise animal health and impact human medicine. In addition to AMU, the role of farm management is an area of growing interest and represents an alternative route for possible interventions. The aim of this study was to evaluate the impact of farm management practices and AMU on resistances of sentinel bacteria in bulk milk. Dairy farms from two, geographically separate locations within the British Isles were recruited as part of two study groups. Farm management data from study group 1 (n = 125) and study group 2 (n = 16) were collected by means of a face-to-face questionnaire with farmers carried out during farm visits. For study group 2, additional data on AMU was collated from veterinary medicine sales records. Sentinel bacterial species (*Enterococcus* spp. and *E. coli*), which have been reported to be of value in antimicrobial resistance (AMR) studies, were isolated from bulk tank milk to monitor antimicrobial susceptibilities by means of minimum inhibitory concentrations (MICs). MIC data for both groups was used to generate an overall "score" for each farm. For both groups, this overall farm mean MIC was used as the outcome variable to evaluate the impact of farm management and AMU. This was achieved through use of elastic net modelling, a regularised regression method which also featured a bootstrapping procedure to produce robust models. Inference of models was based on covariate stabilities and bootstrapped P-values to identify farm management and AMU practices that have significant effects on MICs of sentinel bacteria. Practices which were found to be of importance with respect to *Enterococcus* spp. included management of slurry, external entry of livestock to the dairy herd, use of bedding materials and conditioners, cubicle cleaning routines and antibiotic practices, including use of β-lactams and fluoroquinolones. Practices deemed to be of importance for *E. coli* MICs included cubicle and bedding management practices, teat preparation routines at milking and the milking procedure itself. We conclude that a variety of routine farm management practices are associated with MICs of sentinel bacteria in bulk milk. Amendment of these practices offers additional possible routes of intervention, alongside alterations to AMU, to mitigate the emergence and dissemination of AMR on dairy farms.

1. Introduction

Antimicrobial resistance (AMR) is a major concern in human medicine and also a growing concern in veterinary medicine (Stevens et al., 2018). Antimicrobials play an important role in the care of food producing animals since they are essential for maintaining health in the treatment of bacterial diseases. Healthy, productive animals are considered likely to provide high quality food for human consumption at a lower cost (Oliver et al., 2011).

The use of antimicrobials in agriculture, however, places a selection pressure on pathogenic and commensal bacteria (Chantziaras et al., 2014). This poses a risk for the emergence and dissemination of antimicrobial resistant bacteria as well as the transfer of resistance related genes between bacterial species and populations (Palma et al., 2020). These may be passed on down the food chain and potentially compromise human health (Paphitou, 2013). The potential risk for the

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emergence and dissemination of AMR related genes between bacteria and amongst hosts warrants judicious monitoring of AMR. When studying or monitoring AMR in the dairy farm environment, there are a number of options available but it has been suggested that bacteria isolated from milk samples taken from the bulk tank may be of particular value (Berge et al., 2007). The bulk tank presents a convenient reservoir for sampling which is accepted to be representative of the herd population and its environment. In addition, bacteria present in milk may pose a risk for human consumption (Del Collo et al., 2017), which may be compounded by resistant strains, making the testing of bulk tank milk important. Many studies on dairy farms report using resistance of bacteria isolated from bulk tank milk, across a range of bacterial species, as a proxy of farm level resistance patterns (Kreausukon et al., 2012; Del Collo et al., 2017).

Enterococcus spp. and *Escherichia coli* are commonly used as sentinel bacterial species used in AMR monitoring studies (Borck Høg et al., 2016). *Enterococcus* spp. allow monitoring of resistance in Gram positive bacteria, with *E. coli* allowing for monitoring of resistance in Gram negative bacteria. These bacterial species are used when investigating resistance for a number of reasons; they are ubiquitous in the environment of livestock, they form part of the host microbiota in the gastrointestinal tract and can rapidly acquire resistances to a range of antimicrobial agents with the ability to disseminate these via vertical and horizontal transmission (Borck Høg et al., 2016).

Data relating to antimicrobial use (AMU) on farms are useful when considering AMR as they highlight how antimicrobials of interest have been used and provide an insight into how patterns of usage may change over time (Redding et al., 2019). A number of published studies have found associations between AMR in *E. coli* and patterns of antibiotic use (Saini et al., 2012; Catry et al., 2016). In addition to AMU, the influence of farm management system (conventional vs organic) on AMR has been acknowledged and it is suggested that management is the most important factor related to resistance after AMU (Murphy et al., 2018). These authors noted that although conventional and organic systems were identified as a point of importance, these systems may represent a range of many practices relating to farm management, such as housing, biosecurity and farm density. Such factors may play an important role in the emergence of AMR, either through direct associations or indirectly, by encouraging increased AMU.

Therefore, to fully understand AMR on-farm, simultaneous investigation of both AMU and general farm management policies is needed. The aim of this study was to evaluate the associations between farm management practices and AMU on the resistance of sentinel bacteria in bulk milk. Farm data were collected during face-to-face interviews from two dairy herd populations and resistance was measured using minimum inhibitory concentrations (MICs) of *Enterococcus* spp and *E. coli*.

2. Materials and methods

The study was approved by the School of Veterinary Medicine and Science Ethics Committee (no: 2162 171128).

2.1. Study populations

Farms to be included were sourced from two, geographically distinct regions within the British Isles. Study group 1 consisted of 125 dairy farms located across England and Scotland used in a previous research study (Bradley et al., 2018). Farms were recruited on the basis of bedding material used in dairy cow housing; recycled manure solids (RMS), fresh sand and sawdust. The aim was to recruit a minimum of 40 farms using either of these materials, with farmers being approached via contacts made previously by the research team, veterinarians and participating farmers. Farms to be recruited were additionally matched according to milking method (conventional or automated) and geographic location (East/West UK). Detailed recruitment of farms is described by Bradley et al. (2018). Study group 2 consisted of 18 dairy herds that comprised the whole population of a closed, island location. Farmers were approached for study recruitment via existing contacts, of which 16 agreed to take part.

Data from farms comprising study group 1 had been collected as part of previous research regarding bacteriology of bedding materials used in dairy cow housing. Data collection for study group 2 was collected prospectively, specifically for this research. Study group 1 data were included to allow comparison with findings from study group 2. Methods regarding bacteriology and susceptibility testing were not identical between studies since the two were independent and carried out at different times. The principles of data collection, sample handling and analyses were the same for both studies.

2.2. Farm management questionnaire

Questionnaires were developed to capture a broad range of management practices potentially associated with AMR. The questionnaires featured mainly multiple choice and closed questions within the following main themes; livestock population and herd status, drinking water sources for dairy stock, milking procedures and dairy cow housing including scraping down practices, bedding management, bedding materials and the use of chemicals. Development of the questionnaire for study group 2 was based on that used for study group 1, although additional detail was captured for some areas of farm management. Additional details related to dry cow management, mastitis treatments, disease control strategies, calf rearing and slurry management. The questionnaire also provided for the farmer to comment on any changes in management routines which occurred within the previous 12 months, ensuring this information was also captured. An overview of both questionnaires are included in supplementary material (Tables S1 and S2).

For both study groups, data were collected by means of a face to face interview with farmers during dedicated farm visits. Data from study group 1 was collected during farm visits carried out by five members of a dairy consultancy organisation between December 2014 and March 2015 with each farm being visited once by one consultant. For study group 2, questionnaires were conducted between January and April 2019 by the author (DM).

All data were entered into a spreadsheet (Microsoft Excel, Microsoft Corporation, 2016). Data were checked for outlying or implausible values, but none requiring removal were identified. Questions that were multiple choice but could have multiple answers were given numeric codes for the purpose of analysis.

2.3. Antimicrobial use data

In addition to farm management, data relating to AMU were retrieved at the time of farm visits and collated from veterinary records of antimicrobial sales by the author (DM) for farms comprising study group 2. These data were used as a proxy for AMU on farm between January 2018 and April 2019. Consent to access records was obtained from farmers and sales data were exported from veterinary practice software used for generating invoices for clients, guaranteeing a high accuracy of recording. For some farms, additional sales records were retrieved where online sales had taken place. All sales data were filtered by date and treatment type (antimicrobial) and then exported to Microsoft Excel (Microsoft Corporation, 2016). Raw output data were cleaned and refined by removing unnecessary information regarding client, veterinary surgeon and clinical details as well as financial details. Records for all antimicrobials dispensed by the veterinary practice during this time were categorised by active ingredient, antimicrobial class and the total amount of active antimicrobial ingredient calculated (in grams). AMU data to be included in final analysis was calculated on a per cow basis, taking into account the herd size of each farm.

2.4. Recovery of bacteria from bulk tank milk

For study group 1, a 500 ml milk sample was collected on the day of the farm visit (these occurred during the period between December 2014 and March 2015). Samples were taken either from the top of the bulk tank or from the milk tank outlet following drainage of milk. All samples were packed immediately in insulated boxes with icepacks and dispatched to the laboratory (Quality Milk Management Services Ltd, Wells, Somerset) for bacterial isolation and culturing. Milk samples taken from each farm were plated on the following media;.

- Columbia (5% sheep blood) Agar (Biomerieux): 10 μl spread and incubated for 18–24 h at 37 °C (±2 °C).
- MacConkey Agar (Biomerieux): 100 μl spread and incubated for 18–24 h at 37 °C (±2 °C).
- Violet Red Bile Agar (Acumedia): 100 μl spread and incubated for 18–24 h at 37 °C (±2 °C).
- Slanetz and Bartley Agar (Oxoid) 10 μl and 100 μl spread and both plates incubated for 44–48 h at 35 °C (±2 °C).

MacConkey and Violet Red Bile agar were used in the isolation of *E. coli*, with Slanetz & Bartley agar being used for the isolation of *Enterococcus* spp. Columbia (5% sheep blood) agar was used as a non-selective comparison. A minimum of three *E. coli* and three *Enterococcus* spp. colonies (based on morphology) were selected for pure plating on Columbia (5% sheep blood) agar and incubated for 18–24 h at 37 °C. Isolate IDs were confirmed by MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) (MALDI Biotyper 3.1, Bruker Daltonics, Coventry, UK). Isolated organisms were suspended on glycerol beads and stored at −80 °C using the Protect Microorganism Preservation System (Technical Service Consultants Ltd, Heywood, UK) until ready for susceptibility testing.

For study group 2, five frozen milk samples from the bulk tank from each of the study herds (approximately 500 ml each), taken between August 2018 and November 2019 were used in our analysis. Milk was received frozen due to transit to mainland Britain. Any changes in farm management routines prior to farm visits (previous 12 months) were accounted for in the questionnaire, but an assumption was made that no significant changes in routines would be made between questionnaire completion and the end of bulk tank sampling. Bulk milk samples were received and processed on the day of delivery with bacteria being recovered from milk fat (identified as an enriched culture medium). For all samples, *Enterococcus* spp. and *E. coli* were cultured using selective media (Slanetz & Bartley (SB) and Tryptone Bile X-Glucuronide (TBX) agar respectively). Once defrosted, the milk was pre-incubated for two hours at 37 °C. Following incubation, samples were inverted to allow the milk to mix. For each farm, 10–12 ml of milk was transferred into three sterile falcon tubes which were then centrifuged for two minutes at 4000 rpm. Following centrifuge, milk fat from each falcon tube was spread across two plates (SB or TBX). Approximately 300 μl of milk supernatant were added to each plate to allow for a consistent spreading of the fatty constituents. The contents of each plate were mixed using a spreader to create a smooth consistency and then spread evenly in order to enhance growth across the whole plate. Once dry, plates were incubated for 48–72 h at 44 °C and checked after 48 h for growth. At 48 h, any plates that lacked growth were discarded. Plates that did not have significant bacterial growth were left until 72 h had elapsed and rechecked. Plates that featured significant growth of contaminants (i.e. where two or more contaminant colonies were identified morphologically) were discarded. Eight to ten colonies of *Enterococcus* spp. and *E. coli* per farm were selected from the six SB or TBX plates. Colonies were selected by visual assessment of morphology. When there was growth across all six plates, colonies where selected from all plates to obtain a variety of strains. MALDI-TOF MS was subsequently used to confirm species identification. Colonies that were identified as being either *Enterococcus faecalis, Enterococcus faecium, Enterococcus durans* or *E. coli* were selected for pure plating. At least six colonies of either *Enterococcus* spp. or *E coli* were required to be cultured from a bulk milk sample to be carried forward to susceptibility testing. If six colonies could not be obtained, milk samples were handled according to the method previously stated, using milk remaining in the 500 ml sample which had been stored frozen. If no attempts to recover at least six *Enterococcus* spp. or *E. coli* colonies were successful, then a further milk sample was requested for processing at the earliest possible date. The aim for isolating at least six colonies was to achieve a reasonable spectrum of bacteria from each farm's bulk tank sample. Following recovery, isolates were pure plated on Columbia (5% sheep blood) agar and placed in cold storage with isolates being subsequently suspended on glycerol beads and stored at −80 °C until ready for susceptibility testing.

2.5. Antimicrobial susceptibility testing

Bacterial isolates were pure plated from the stored glycerol beads onto Columbia (5% sheep blood) agar and incubated for 18–24 h at 37 °C.

For study group 1, antimicrobial susceptibilities were determined using a VITEK® 2 (Biomerieux; Basingstoke UK) according to manufacturer's instructions. Testing was carried out during June and July 2015. VITEK® 2 AST GN65 and GP76 cards were used for determining *E. coli* and *Enterococcus* spp. MICs respectively.

Antimicrobial susceptibilities for isolates obtained from study group 2 were determined using Thermofisher's Sensititre Antimicrobial Susceptibility Testing System (Thermo Scientific; Massachusetts, USA) Sensititre COMPGN1F AST cards were used to test both *E. coli* and *Enterococcus* spp. antibiotic susceptibilities according to the manufacturer's recommended protocol. Following inoculation, AST cards were incubated at 35 °C for 18–20 h. Following incubation, the plate was read using the Sensititre Vizion and SWIN™ software. Further susceptibility testing using Micronaut-S Mastitis 3 (Merlin; Bornheim-Hersel, Germany) AST plates was carried out via the same procedure outlined above to provide a broader range of susceptibility profiles. All susceptibility testing was carried out between October and December 2019. All data were entered into a spreadsheet (Microsoft Excel, Microsoft Corporation, 2016). Isolates were determined as being either susceptible or resistant according to clinical breakpoints established by the Clinical and Laboratory Standards Institute (CLSI). Where CLSI breakpoints were not available, interpretative criteria provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used. Multidrug resistance was determined where breakpoints were available. This was defined as isolates resistant to ≥ 1 antibiotics from ≥ 3 separate antibiotic categories, excluding those known to show intrinsic resistance. Antimicrobials which showed no variation in MICs during the sampling period were omitted from analysis, which also included those to which the bacteria showed intrinsic resistance. Antimicrobials included in final analysis are listed in supplementary material (Tables S3-S6).

2.6. Antimicrobial susceptibility scoring

All MIC data for study groups 1 and 2 were used to generate an overall resistance score for each bacterial species for each farm. This was done by calculating the mean MIC of all antibiotics selected for analysis for both *Enterococcus* spp. and *E. coli* across the sampling period (August 2018 to November 2019). To overcome the differing MIC scales of each antibiotic tested when calculating the overall mean, each MIC data point was first rescaled from tested concentration ranges to a standardised scale. This meant the MIC values for all antibiotics were rescaled to cover the same range. This procedure was based on the number of microdilutions of each antibiotic used and calculated according to the following equation;

$$\left(\frac{No.\ of\ microdilutions\ of\ highest\ frequency\ antibiotic}{No.of\ MICs\ within\ antibiotics\ tested\ range}\right) \times No.\ of\ microdiluations\ constituting\ the\ MIC$$

The overall farm MIC score allocated to each bulk tank sample differed slightly between study groups. For study group 1, a mean of standardised MIC values for antimicrobials tested against both *Enterococcus* spp. and *E. coli* was attributed to a single bulk tank milk sample collected from each farm between December 2014 and March 2015. For study group 2, the mean standardised MIC for antimicrobials tested against both sentinel bacterial species across six samples taken from each bulk tank sample obtained between August 2018 and November 2019 was used to provide an overall MIC score. For both groups, this overall farm mean MIC was used as the outcome variable to evaluate the impact of farm management and AMU on resistance to *Enterococcus* spp. and *E. coli*.

2.7. Data analysis

Initial descriptive analysis was conducted separately for farms in study groups 1 and 2, to identify key patterns within the data. To avoid overfitting due to the large number of potential explanatory variables relative to the number of observations (herds), regularised regression with stability selection was conducted for inference (Zou and Hastie, 2005; (Meinshausen and Bühlmann, 2010). Explanatory variables were coded as numeric or categorical and numeric covariates were standardised to a common scale, by subtracting the mean and dividing by twice the standard deviation, as previously reported (Gelman, 2008).

Regularisation was carried out using a linear elastic net regression model with a continuous outcome using the "glmnet" and "caret" packages (Friedman et al., 2010; Kuhn et al., 2018) within the R statistical software platform (R Core Team, 2021). Elastic net regression combines the effects of ridge and lasso regression (Zou and Hastie, 2005). Penalised maximum likelihood was used to fit models with a cyclical coordinate descent algorithm to conduct parameter estimation via algorithms which solve the equation through cyclical coordinate descent (Friedman et al., 2010). Elastic net models constructed for both study groups took the following form;

$$SSE_{enet} = \frac{1}{2n}\sum_{i=1}^{n}(y_i - \hat{y}_i)^2[\boldsymbol{\lambda}]\sum_{j=1}^{P}\frac{1}{2}(1-\boldsymbol{\alpha})\beta_j^2 + \alpha\beta j$$

where SSEenet represented the elastic net loss function to be minimised, i denoted each observation and n the number of observations (farm), y_i was the observed outcome and $\hat{y}_i$ the predicted outcome, λ was the penalisation parameter, j denoted a predictor variable; p denoted the number of predictor variables in total, α was a mixing parameter that defined penalisation on either the sum of the square of the coefficients (β^2) or the unsquared absolute value of coefficients (β).

The optimal values of tuning parameters alpha and lambda for all models were determined using five-fold cross validation, repeated 20 times, to identify values that minimised the mean absolute error (MAE) (Kuhn and Johnson, 2013).

To estimate covariate stability and P-values, a bootstrapping procedure was undertaken to ensure robust estimation of model parameters (Hastie et al., 2015; Lima et al., 2020). In brief, this comprised using a bootstrapping procedure to rerun elastic net models 500 times. Model parameters from each bootstrapped sample were stored in a matrix and used for inference. Final inference was based on two main outcomes - parameter stability and a bootstrapped P-value. Parameter stability refers to the percentage of times that a particular variable was selected in the model across the 500 bootstrap samples; the higher the percentage, the less likely the covariate is to be a false positive result (Meinshausen and Bühlmann, 2010). The 'Bootstrap P value' (BPV) was calculated as the minimum proportion of (non-zero) coefficient values to one side of zero. That is, if a covariate was selected in the model in 400 of the bootstrap samples and 390 of these had a value either greater or less than zero, then the Bootstrap P value would be (400−390)/400 = 0.025. Covariates were selected in the final model and deemed 'significant' when both BPV <0.05 with a high covariate stability. These thresholds were identified by plotting stabilities against significance (supplementary material Figs. S1–S3). *Enterococcus* spp. and *E. coli* model stabilities for study group 1 were defined as ≥ 80% and ≥ 75% respectively, while the *Enterococcus* spp. stability for study group 2 was defined as ≥55%.

3. Results

3.1. Population characteristics

The final dataset for study group 1 comprised 94 farms with information relating to *Enterococcus* spp. MICs and 87 farms relating to *E. coli* MICs. Herd size ranged from 110 to 1550 adult cows, with a mean herd size of 358 and a median of 290 cows. For the sixteen farms comprising study group 2, herd size ranged from 10 to 280 adult cows with a mean herd size of 151 and a median of 183.

3.2. MIC distributions

For study group 1, final analysis included 171 *E. coli* isolates and 293 *Enterococcus* isolates (*E.faecalis;* n = 93, *E.faecium; n* = 107, *E.durans; n* = *93)*. Data pertaining to the percentage of isolates deemed resistant and the distribution of MICs are presented in supplementary material (Tables S7–S9). Multidrug resistance for *Enterococcus spp.* and *E. coli* was found to be 6.8% and 11.1% respectively.

For study group 2 (sampling period August 2018 – November 2019), 365 *Enterococcus* spp. (*E.faecalis;* n = 249, *E.faecium; n* = 97, *E.durans; n* = *19*) and 451 *E. coli* were isolated from milk samples. The percentage of these isolates deemed resistant alongside MIC distributions are presented in supplementary material in Tables S10 and S11. Multidrug resistance for *Enterococcus spp.* and *E. coli* was found to be 1.4% and 1.3% respectively.

3.3. Statistical models

The final bootstrapped elastic net regression models built for study group 1 data (*Enterococcus* spp. and *E. coli)* and study group 2 (*Enterococcus* spp. only) are provided below. The MIC data for *E. coli* for study group 2 displayed exceptionally low variability between farms and therefore was unsuitable to produce a robust model.

3.3.1. Study group 1; Enterococcus spp.

Results of the final model for management factors associated with *Enterococcus* spp. MICs for study group 1 are presented in Table 1. Covariates selected in the final model related to the size of milking parlour, farm location, use of automatic milking systems and practices associated with bedding materials. Farms with parlours containing between 13 and 24 units and those between 25 and 36 milking units had higher MICs than farms with smaller parlours of ≤ 12 milking units. In terms of geographic location, farms in the north west of England had higher *Enterococcus* spp. MICs when compared to farms elsewhere in the country. Farms with automated milking systems had higher MICs than those where cows were milked conventionally. Practices associated with cubicle bedding were selected in the final model with farms using recycled manure solids (RMS) having increased MICs compared to those using sawdust. A decreased frequency of cubicle bedding was associated with lower MICs in *Enterococcus* spp.

3.3.2. Study group 1: E. coli

Results of the final model for management factors associated with *E. coli* MICs for study group 1 are presented in Table 2. Bedding of cubicles once daily was associated with significantly lower MICs for *E. coli*

Table 1
Final elastic net regression model for farm management practices, in order of descending covariate stability, associated with changes in MIC of *Enterococcus* spp. from bulk tank milk samples for study group 1 (n = 94 farms). Covariate stability threshold for variable selection was > 80%.

Variable	No. of observations in category	Reference category	No. of reference observations in category	Covariate stability (%)	Coefficient	Bootstrap P-value
No. of parlour units 13–24	42	No. of parlour units ≤ 12	12	97	0.17	0.03
Farm location – North West England	33	Farm location – East England	7	92	0.18	0.03
No. of parlour units 25–36	16	No. of parlour units ≤ 12	12	88	0.27	<0.01
Automated milking	6	Conventional parlour milking	63	87	0.35	0.02
Bedding material – RMS	29	Bedding material – sawdust	34	84	0.21	0.01
Cubicles bedded once per week or less frequently	13	Bedding cubicles twice per day	17	83	-0.32	0.04

Table 2
Final elastic net regression model for farm management practices, in order of descending covariate stability, associated with changes in MIC of *E. coli.* from bulk tank milk samples for study group 1 (n = 87 farms). Covariate stability threshold for variable selection was > 75%.

Variable	No. of observations in category	Reference category	No. of reference observations in category	Covariate stability (%)	Coefficient	Bootstrap P-value
Bedding cubicles once daily	41	Bedding cubicles twice per day	13	90	-0.06	0.01
No use of bedding conditioners on cubicles	47	Bedding conditioners used on cubicles	39	87	-0.06	0.02
Milk sales (litres/cow/year)[a]	–	–	–	84	0.06	<0.01
Teats brushed before milking	11	Teat preparation with pre milking disinfectant	52	84	0.13	<0.01
Teats wiped with dry cloth before milking	11	Teat preparation with pre milking disinfectant	52	80	-0.11	<0.01
Automatic milking	6	Conventional parlour milking	57	78	-0.06	0.01

[a] Standardised variable; coefficient relates to change of one unit on a standardised scale.

compared to farms that bedded cubicles twice daily. Significantly lower MICs were identified on farms that did not use bedding conditioner materials on cubicles compared with farms that did. Milk yield was found to be important; increasing yields (litres produced per cow per year) were associated with significantly increased MICs. Milking preparation procedures involving teat brushing resulted in significantly increased MICs, whereas the wiping of teats with dry cloths or towels resulted in significantly reduced MICs compared to the use of pre-milking teat disinfection without brushing. Milking system was again found to be important, with farms using automated milking systems being associated with significantly lower MICs than those where cows were milked in a conventional parlour.

3.3.3. Study group 2: Enterococcus spp.

Results of the final model for management factors and antibiotic use associated with *Enterococcus* spp. MICs for study group 2 are presented in Table 3. The presence of a slurry store on farm was found to be important; farms without slurry stores had significantly lower MICs than those with a store. Farmers who purchased antimicrobials online had *Enterococcus* spp. isolated from bulk milk with significantly higher MICs than those who purchased medicines from their veterinary practice only. Several factors relating to cubicle management were found to be important. Farms where hydrated lime was used on cubicles as an antibacterial product resulted in a significantly higher MIC than those that did not use any antibacterial products. For farms where bulls used for breeding were reared on farm rather than being borrowed or purchased, significantly lower mean MICs were identified. Farms that did not practice 'natural' drying off (i.e. always used either antibiotic therapy or teat sealants) had a significantly higher mean MIC than those farms where natural drying off was practiced.

Antimicrobial classes identified from veterinary sales records were; aminocoumarin, aminoglycoside, β-lactam, cephalosporin, fluoroquinolone, lincosamide, macrolide, sulfonamide/trimethoprim and tetracycline. The use of two classes of antimicrobials were found to be of

Table 3
Final elastic net regression model for farm management and antibiotic use practices, in order of descending covariate stability, associated with changes in MIC of *Enterococcus* spp. from bulk tank milk samples for study group 2 (n = 16 farms). Covariate stability threshold for variable selection was > 55%.

Variable	No. of observations in category	Reference category	No. of reference observations in category	Covariate stability (%)	Coefficient	Bootstrap P-value
No slurry store present on farm	5	Slurry store on farm	11	81	-0.03	<0.01
Medicine purchase from vet & online	5	Medicine purchase from vet only	10	71	0.07	<0.01
Breeding bulls reared on farm	4	Some or all breeding bulls brought into herd	3	65	-0.03	<0.01
β-lactam use more than 2.5 g/cow	4	β-lactam use less than 1 g/cow	4	61	0.03	<0.01
Fluoroquinolone use more than 0.2 g/cow	3	Zero use of fluoroquinolone	7	60	0.06	<0.01
No natural drying off of cows	13	Natural drying off occurs	3	59	0.022	<0.01
Hydrated lime used on bedding	6	No antibacterial used	6	57	0.031	<0.01

importance in the model; higher levels of β-lactam and fluoroquinolone usage were associated with significantly higher MICs in *Enterococcus* spp.

4. Discussion

The contribution of AMU to the emergence of AMR is important and widely recognised (Hommerich et al., 2019). In the context of livestock agriculture, as well as AMU, other factors may be of important for the emergence of AMR and should be considered, including the contribution of farm management practices (Murphy et al., 2018). The aim of this study was to identify farm management factors that most influence MICs in sentinel bacterial species isolated from farm bulk tank milk samples. These factors may provide a basis for potential on-farm interventions to help limit increases in MICs of important bacterial species within the farm environment (Murphy et al., 2018).

The MIC is a measure of the lowest concentration of an antimicrobial needed to inhibit growth of microbes, such as bacteria (Ericsson and Sherris, 1971). For ease of interpretation, raw MIC data may be categorised according to epidemiological cut-off values or by clinical breakpoints (Michael et al., 2020). Susceptibilities of bacterial isolates are most commonly categorised as either susceptible, intermediate or resistant. This may however, lead to a loss of information by disregarding the distributions of raw data. It has been shown that utilisation of breakpoints in analysis fail to identify important developments in resistance distributions between instances of sampling and testing (Lindeman et al., 2013; Mazloom et al., 2018). Therefore, the avoidance of MIC data categorisation in the context of this study may allow for subtle changes in MICs to be detected and to better identify factors associated with these changes. This may allow for interventions to be made to prevent bacterial susceptibilities reaching critical points of clinical significance e.g. bacterial isolates moving from intermediate to resistant.

A number of management factors were identified to be associated with a net increase or decrease in MICs in *Enterococcus* spp. and *E. coli* across study farms. These factors covered a range of areas, such as slurry management, cubicle bedding, teat management at milking as well as frequency of milking, dry cow management and entry of animals onto farm from elsewhere. The threshold of covariate stability for study group 1 was implemented at $\geq$75% and $\geq$80%, while for study group 2 a covariate stability of $>$55% was used. The threshold selected was based on graphical inspection of covariate stabilities and bootstrap P values (Lima et al., 2021). The small sample size of study group 2 farms reduced the statistical power available and it is unsurprising that covariate stability was lower. Although there may be less certainty of the true effect of covariates with lower stability (Meinshausen and Bühlmann, 2010), they still may be associated with the outcome variable. Since this study is cross-sectional in design, verification of causality for all covariates identified in final models is important to establish in future research and in this respect, the associations identified in this study should be interpreted with caution.

The importance of slurry in the context of antimicrobial susceptibilities was identified for study group 2. In this study we found that on farms where there were no slurry stores, there were lower MICs compared to farms where stores were in use. This refers to the storage in above ground structures of animal waste during a period when spreading of slurry on land is prohibited between October and February due to environmental concerns. Outside of this period, slurry may be spread on farmland. Waste is moved from slurry tanks beneath housing to these storage units when they become full, and represents a period of long term storage. Farm animal manure has been identified as a significant reservoir of antimicrobial compounds, resistant bacteria and antibiotic resistant genes (Heuer et al., 2011). Slurry storage is noteworthy as it facilitates an environment with the potential to encourage AMR to emerge and spread (Lanyon et al., 2021). Mathematical modelling using parameters identified in previous work, along with incorporating these into their own parameters, evaluated the role of slurry storage in AMR (Baker et al., 2016). The authors reported that the proportion of bacteria showing AMR characteristics increased throughout the storage period as a result of horizontal gene transfer and by selection of resistant genes. Our study presents results similar to previous findings and suggest the role of slurry storage may be important in contributing to increased MICs on farm. Importantly, the spreading of stored slurry onto land used for grazing and silage may represent a potential route for transmission of resistant organisms to dairy cows and perpetuate their existence in the farm environment.

Results from study group 2 indicated that the use of antibacterial materials on cubicle bedding to be important with regards to *Enterococcus* spp. isolated from farms in this group. The use of hydrated lime was associated with increased MICs, whereas decreased MICs were seen on farms that did not use any antibacterial products on cubicles. Additionally, as identified for *Enterococcus* spp., the use of antibacterial bedding conditioners (including hydrated lime) in study group 1 was associated with increased MICs in *E coli*. It has been reported that the use of antibacterial materials, such as lime based products, significantly reduce bacterial counts in bedding and on cow teats (Janzen et al., 1982; Paduch et al., 2013). The association found in this study, between the use of antibacterial products on bedding and increased MICs, may be a result of an increased selection pressure on the bacterial populations present in cubicle bedding. This may inadvertently encourage selection for genes giving rise to increased MICs. However, the bacterial mechanisms for such gene selection in this context are unclear and warrants further investigation. Furthermore, there may be the possibility of reverse causation occurring in this instance. Hydrated lime may be being used to address already existing mastitis problems, which may in itself be contributing to higher MICs through increased AMU. However, as previously considered, the cross-sectional nature of this study means that only associations are identified and causality cannot be attributed.

Teat management practices prior to milking were also associated with differences in *E. coli* MICs. These were found to be lower when teats were wiped with a dry cloth when compared with pre-dipping with a teat disinfectant, while MICs were higher when teats were brushed compared with pre-milking teat disinfection. In a previous study evaluating resistance in bacteria isolated from bulk tank milk, farms that practised dry wiping at milking were more likely to have lower MICs than farms that didn't practice dry wiping (Kirk et al., 2005). It was postulated that milking cows with wet teats is associated with an increased incidence of mastitis, which had the potential to increase antibiotic use and therefore increased bacterial susceptibilities. The brushing procedure on farms was accompanied by a disinfection regime, which, together, may provide an explanation for these results, but the dynamics of this are not clear.

Practices relating to the management of cubicles and bedding were associated with increases in MICs in *Enterococcus* spp. and *E. coli* isolates in study group 1. Here, the practice of less frequent bedding application on cubicles was associated with lower MICs. However, an overview of the data shows an association between the type of bedding material used in study group 1 and it's application frequency. Therefore, the type of bedding material used may be of greater importance compared to how often fresh material is laid down on cubicles. Additionally, there were higher MICs seen on farms that used recycled manure solids as a bedding material and this may align with the increased MICs associated with slurry storage seen in study group 2. Furthermore, it has been reported that there were significantly higher bacterial counts in RMS bedding, when compared with sawdust or sand (Bradley et al., 2018). Within a larger population of bacteria, there may be more variability of genetic materials (as well as potential for gene transfer) and an increased chance for mutations to appear in the population. The constant recycling of manure solids, despite processing methods designed to reduce the bacterial load, may help to perpetuate this. RMS bedding materials have been found to promote growth of environmental bacteria, namely *Klebsiella pneumonia*, and to a lesser extent, *E. faecium* (Godden et al.,

2008). The issue of AMR with regards to RMS due to the presence of antimicrobial residues and resistance genes has been noted, with varying levels of success across methodologies aiming to reduce their load in RMS materials (Wallace et al., 2018; Zhang et al., 2020). Our results however suggest that the increase in MICs in sentinel bacteria associated with the use of RMS should be an important consideration in its use.

Automated milking systems (limited to study group 1) were shown to be important for both *E. coli* and *Enterococcus* spp. MICs. From our results, farms on which cows were milked in an automated system rather than in a conventional milking parlour had lower MICs for *E. coli*, However, the converse of this effect was seen for *Enterococcus* spp, which had higher MICs on farms with automated milking. The biological reasons for these contradictory findings are unclear, although one possibility could be differences in routes of antibiotic use. AMU has been compared between automatic and conventional milking herds (Deng et al., 2020) with the conclusion that AMU between systems was similar, but routes of treatment varied. Injectable treatments had a higher frequency of application in automatic milking herds, while the converse was seen for intramammary treatments when compared to conventionally milked herds. Differences in treatment type may exert varying degrees of selection pressures amongst commensal bacterial populations. These pressures may be further influenced by the use of certain antimicrobial classes. It is difficult to know whether these findings are relevant to UK dairy farms, particularly as AMU data were not captured for the farms making up study group 1. Subsequent postulation of causality surrounding AMU in this instance is difficult to establish. It is possible that differences in antimicrobial treatment application between farms could be a driver for contrasting resistance patterns. These findings suggest that type of milking system could be important in relation to AMR and highlights this as an area for future consideration.

The collection and collation of AMU data for farms in study group 2 helped to further highlight the importance this has for AMR at the dairy farm level. It was shown that farms with higher levels of use of antibiotics belonging to β-lactam and fluoroquinolone classes of antimicrobials had higher MICs in *Enterococcus* spp. than those with lower levels of use. Decreased MICs in herds which practiced some degree of 'natural' drying off (no use of antibiotic dry cow therapy) is also noteworthy. Many studies and reviews have reported that higher levels of use of antimicrobials in food producing animals does increase the selection pressure for resistance to emerge amongst bacterial populations (Oliver et al., 2011). Across all farms making up study group 2, historic AMU data showed β-lactam and fluoroquinolone class antimicrobials to be the first and fifth most used respectfully in terms of mass (grams). Aminoglycosides, trimethoprim/sulfamethoxazole and cephalosporin antimicrobials made up the majority of other AMU across farms. However, MIC data for antimicrobials in these classes were less variable than those belonging to β-lactam and fluoroquinolone classes, which may be a reason why these antimicrobial classes were not found to be associated with higher MICs in the sentinel bacteria.

Intrinsic resistances to β-lactams in Enterococci have been recognised, as well as low levels against fluoroquinolones (Heimer et al., 2014). Our results appear to suggest that increased use of these antimicrobial classes may increase MICs further. The association between higher levels of β-lactam and fluoroquinolone use and higher MICs may be of particular interest and importance, given the pressure on farmers and veterinarians to become more judicious in their use of certain antimicrobial classes, such as fluoroquinolones and 3rd and 4th generation cephalosporins. A study into antibiotic use on dairy farms between 2005 and 2012 reported that the use of third and fourth generation cephalosporins and fluoroquinolones had fallen from 18% of overall use to only 1%. This reduction however brought about an increase in use of penicillin and other β-lactam products as well as broad spectrum products such as trimethoprim/sulfonamide combinations (Kuipers et al., 2016). Since the use of β-lactam antibiotics may increase in the future, the continued surveillance of antimicrobial susceptibilities to these antibiotics will be critical.

4.1. Study limitations

Study group 1 data was sourced from farms that had been recruited for previous work to evaluate bacterial loads in different bedding materials. Farms were selected with the aim of recruiting at least 40 that used either sawdust, sand or recycled manure solids. Due to this sample selection, it is uncertain how representative these farms may be of farms across Britain. Additional research with the use of true random sampling, should be considered in future to further explore the impact of farm management on patterns of bacterial resistance.

The relatively small sample size of study group 2 means that although the sample represented virtually a whole island population (which is reasonably isolated from mainland Britain), a limitation in statistical power may have meant some management practices of potential importance have been missed. A potential danger with a small sample size when using conventional regression is overfitting of a model. However, the use of the elastic net regression with the additional implementation of stability selection (Zou and Hastie, 2005; (Meinshausen and Bühlmann, 2010) vastly reduces this.

4.2. Conclusions

In conclusion, it has been established that a variety of routine farm management practices are associated with MICs of sentinel bacteria in bulk milk. Although causal relationships are unclear from this cross-sectional analysis, this suggests that changes in farm management may play a role reducing bacterial resistance. Further work to establish to establish causality and identify the most important practices would be of value.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2022.105666.

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