Final report

The association between the use of antimicrobials and resistance in Escherichia coli and Enterococcus species isolated from beef cattle

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Executive summary

Understanding the relationships between antimicrobial use and resistance through surveillance is important to guide antimicrobial stewardship for the Australian feedlot industry. Previous antimicrobial use surveys conducted for Australian feedlot cattle (Badger et al., 2020) have reported use for therapeutic, metaphylactic and prophylactic purposes. Current antimicrobial resistance surveillance programs (Barlow et al., 2022) sample small numbers of grain-fed cattle from numerous abattoirs and deliver insights on resistance at an aggregated population level. Recently, longitudinal studies, such as MLA Project B.FLT.3003 sought to understand the antimicrobial resistance of antimicrobial resistance of *E. coli*, *Salmonella*, and *Enterococcus* species during pre-feedlot, feedlot and slaughter periods a single pen of feedlot cattle.

The use of different antimicrobial classes for treatment, metaphylaxis, and prophylaxis is assumed to be one of the main factors that create selection pressure and contribute to antimicrobial resistance in bacteria from food-producing animals. This study builds on the pilot research conducted in MLA project B.FLT.3003 to further examine the effects of various antimicrobials on the development of antimicrobial resistance in *E. coli* and *Enterococcus* species isolated from 135 sick cattle before treatment with antimicrobials. A sub-set of 63 animals treated with antimicrobials were followed through to slaughter, along with 67 apparently healthy animals. Faecal samples were collected aseptically from rectum of sick cattle just before they received first and/or second treatment across multiple pens in the feedlot. The antimicrobials used for treating sick cattle during the study were in order of frequency: tulathromycin (Draxxin, Zoetis), oxytetracycline (Bivatop 200, Boehringer Ingelheim), and ceftiofur (Excede, Zoetis). Tetracycline-based product (CTC200) and macrolide-based product tilmicosin phosphate (Bovatil 300; South Yarra Pharma) were used for metaphylaxis in cattle arriving from high-risk sources (10 of 11 pens monitored). Finally, faecal swab samples were collected following exit from the feedlot (at the abattoir) from cattle treated for clinical illness (treated cattle) and these not treated for clinical illness (apparently healthy cattle). The target bacteria were isolated and confirmed by culture methods and MALDI-TOF, respectively. The isolates of *E. coli* and *Enterococcus* were tested for resistance to 14-16 antimicrobials, including those used in human and veterinary medicine.

A total of 90 (66.7%) *E. coli* were isolated from 135 samples collected from sick cattle before their first treatment. The highest resistance was observed to tetracycline (80.0%), followed by sulfisoxazole (17.8%), streptomycin (10.0%), ampicillin (5.6%), and azithromycin (5.6%). Additionally, 48 (35.6%) ESBL-producing *E. coli* were isolated from sick cattle before receiving their first treatment. All isolates

were resistant to ampicillin, ceftiofur, and ceftriaxone. At the abattoir, a total of 56 (83.6%) *E. coli* were isolated from 67 rectal swab samples from apparently healthy cattle. The most common resistance was observed to tetracycline (79.0%), ampicillin (15.8%), sulfisoxazole (10.5%) and streptomycin (8.8%). Only one ESBL-producing *E. coli* was isolated and was resistant to ampicillin, azithromycin, ceftiofur, ceftriaxone, streptomycin, sulfisoxazole and tetracycline. Furthermore, a total of 50 (79.4%) isolates of *E. coli* were isolated from 63 rectal swabs sampled from treated cattle. Resistance was observed to tetracycline (54.0%), sulfisoxazole (8.0%), ampicillin (8.0%), and streptomycin (4.0%). Of these samples, 4 (6.3%) ESBL-producing *E. coli* were isolated, and were resistant to ampicillin, ceftiofur, ceftriaxone and tetracycline.

The most prevalent *Enterococcus* spp. isolated from cattle prior to first treatment were *E. faecium* (n=45), *E. hirae* (n=12), *E. durans* (n=11), *E. mundtti* (n=4), *E. raffinosus*, *E. thailandicus* and *E. villorum* (n=1 of each). Among the *E. faecium* isolated from sick cattle before treatment, the highest resistance was observed to tetracycline (71.1%) followed by lincomycin (53.3%), ciprofloxacin (35.6%), erythromycin (26.7%), tylosin (24.4%), quinupristin /dalfopristin (17.8%) and daptomycin (15.6%). The most common *Enterococcus* spp. isolated from apparently healthy cattle (n=67) were *E. hirae* (n=36), *E. faecium* (n=14), *E. durans* (n=3), and *E. mundtti* (n=2). In these *E. faecium (n=14),* resistance was observed to lincomycin (78.6%), nitrofurantoin (50.0%), tigecycline, and ciprofloxacin (14.3% each). In contrast, among the *Enterococcus* spp. isolated from treated cattle at slaughter (n=63), *E. hirae* (n=37) was the most abundant species, followed by *E. faecium* (n=11), E. *durans (n=2),* and *E. sulfureus* and *E. thailandicus* (1 of each). In *E. faecium*, a slightly lower resistance to lincomycin (63.6%) and daptomycin (18.2%) was detected. It may be possible, metaphylaxis increases the risk of AMR in gut bacteria, however further research is required due to the limited numbers of non-metaphylaxis cattle sampled in this study.

In addition to this study, a literature review was delivered on the effect of ionophores on antimicrobial resistance of the microbiome, E coli, Salmonella and Enterococcus in beef cattle and wider food animal production. This review also addressed protozoa. Despite the relatively long history of use for over five decades, the level of reported ionophore resistance is still miniscule. In conclusion of the literature review, the use of monensin (and other ionophores) in beef feedlot cattle was unlikely to have a significant effect on the development and dissemination of antimicrobial resistance determinants of clinical significance from cattle to humans.

Recommendations for future research and surveillance from these study findings include:

1. Continuous surveillance of AMR in feedlot indicator bacteria is essential, both pre-treatment and at slaughter
2. Of particular importance are
    a. The 3rd generation cephalosporin resistance in *E. coli* isolated from cattle pre-treatment that decreases over time to low prevalence at slaughter
    b. Daptomycin, Quinopristin / Dalfopristin and Nutrofurantoin resistances in enterococci, including genetic mechanisms of their resistance
3. Whole-genome sequence of all resistant and representative control isolates from this project is essential to
    a. Compare them with the pig, poultry, and human clinical isolates from Australia
    b. Potentially detect novel mechanisms of resistance in isolates with high disproportion between genotypic (detected by whole genome sequencing) and phenotypic resistance (already established during this project)
4. Studies of how antimicrobial resistance is acquired from the environment
5. Larger sample size from multiple feedlots and pens should be included in the future research
6. The industry should continue to focus on implementation of the antimicrobial stewardship guidelines
7. The use of metaphylaxis in feedlot industry should be minimised

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1. Project objectives

1) Conduct a literature review on the effect of ionophores and bambermycins on antimicrobial resistance of the microbiome, E coli, Salmonella and Enterococcus in beef cattle and wider food animal production. This review will also address protozoa.

(2) Determine through experimentation in the feedlot industry, the effect of antimicrobial use on longitudinal resistance of hospital cattle.

2. Background

Understanding the relationships between antimicrobial use and resistance through surveillance is important to guide antimicrobial stewardship for the Australian feedlot industry. It is also important to build objective data to understand resistance mechanisms in beef cattle to guide further risk-based modelling for food safety. The emergence of antimicrobial resistance in susceptible bacteria occurs either through mutation and/or horizontal gene transfer (HGT). The existing resistant bacteria increase with exposure to antimicrobials (Bergman et al., 2009). Antimicrobials are routinely used in human and veterinary medicine to treat and/or prevent diseases. Hence, high resistance rates significantly reduce the chance of effectively treating infections in both humans and animals. Use of antimicrobials can potentially lead to selection of antimicrobial resistance (AMR) among bacterial populations within the treated human/animal (Witte, 1998) in both commensal and pathogenic microbes. The resistance in commensal enteric bacteria is generally correlated with pathogenic bacteria (Bag et al., 2019). The alimentary (digestive) system is suitable for developing and disseminating antimicrobial resistance in enteric bacterial pathogens (Schjørring and Krogfelt, 2011). At present, there is concern that antimicrobials used in animals could increase the risk of dissemination of resistant zoonotic pathogens and, in turn, resistant genetic determinants being transferred to human pathogens e.g. Shiga-Toxin *Escherichia coli* and *Salmonella enterica* strains. There is however, limited objective data to support horizontal gene transfer between bacteria in beef cattle and humans, however there are documented cases of horizontal gene transfer for colistin and avoparcin resistance in swine and poultry, and plasmid mediated streptogramin resistance in poultry (Cho et al., 2022; Hammerum, 2012; Webb et al., 2017). Risk of transmission must first account for the prevalence of the bacterial shedding and transfer to meat-based products. Secondly, bacteria must survive cooking, digestive processes and establish themselves in the human intestinal tract, prior to transfer of their resistance determinants between sub-species and serotypes. Furthermore, resistance determinants need to be stably retained if they are transferred between bacteria. As a

result, it is very important to survey resistant bacteria along the stages of the food chain and estimate risk factors contributing to the development and dissemination of AMR bacteria. Australia has strict registration and regulation of antimicrobial use in livestock production systems. This minimises the risk of development and spread of AMR to the critically important antimicrobials used in human clinical practice. Despite these restrictions, there is a need for ongoing surveillance of AMR in bacteria that may cause clinical infections in humans and also frequently colonise the gut of livestock. *Enterococcus* species and *Escherichia coli* are bacteria of concern associated with gut colonisation that could be potentially transferred to humans through the food chain. Monitoring AMR in commensal microbes can contribute to the understanding of the selection process mediated by antimicrobial used in a feedlot setting and how it contributes to the overall resistance burden in these commensal bacteria.

This study examined the effects of the use of various antimicrobials on the development of antimicrobial resistance in *E. coli* and Enterococcus species isolated from beef cattle as a follow on study from MLA Project B.FLT.3003. The previous study reported in *Enterococcus faecium* at slaughter high levels of resistance to lincomycin and nitrofurantoin; moderate levels of resistance to daptomycin and quinpristin/dafloprístin; and low levels of resistance to ciprofloxacin. These findings are of concern as both daptomycin and quinpristin-dafloprístin are antimicrobials of last resort in sepsis treatment for this bacterium in Australian hospitals. No vancomycin resistance was detected in any *Enterococcus faecium* which was a positive finding. For *E. coli* moderate levels of resistance at slaughter were observed for tetracycline, followed by low levels of resistance to ampicillin, streptomycin, sulfisoxasole, and ceftiofur (4.4%). MLA Project B.FLT.3003 was carried out in a single pen of feedlot cattle with low usage rates of antimicrobials (8.7% treatment rate). This study was conducted at the same feedlot across multiple pens and investigated resistance of isolates cattle entering the hospital and at slaughter compared to cattle that received no antimicrobials.

2. Methodology

2.1. Study animals and sample collection

A study was conducted to determine the AMR status of *E. coli* and *Enterococcus* spp., isolated from beef cattle in Southern Australia from May to August, 2021. Samples were collected from two groups of cattle, **treated cattle** (cattle being treated with therapeutically with antimicrobials for clinical illness during the feedlot stay in the hospital pen originating from 11 different pens) and **apparently healthy cattle** (cattle not being treated therapeutically for clinical illness during the feedlot stay originating

from the same 11 pens as treated cattle). Antimicrobials used for the treatment of treated cattle were tulathromycin (Draxxin, Zoetis), oxytetracycline (Bivatop200, Boehringer Ingelheim) and ceftiofur (Excede, Zoetis). Treated cattle were transferred to the hospital pen for treatment and returned to their pen when clinically recovered. Concurrently, tetracycline-based product (chlortetracycline; CTC200) and macrolide-based product tilmicosin (Bovatil 300, South Yarra Pharma) were also used for metaphylaxis of cattle arriving in the feedlot from high-risk sources (e.g., cattle bought from sale-yards). Only one of these 11 pens was not exposed to any metaphylaxis.

Approximately 15g of faeces was collected from the rectum of the treated cattle just before they received first and/or second treatment. Additionally, faecal swab samples were collected following exit from the feedlot (at the abattoir) using Ames transport media swabs (Copan, Italy) from treated and apparently healthy cattle. These samples were obtained post-evisceration by incision into the rectum 15–30 cm cranial to the anus following the method described by Abreham et al. (2019). The faecal samples were transported to the laboratory under chilled conditions in EPS box containing frozen gel packs. Of the 465 faecal samples collected at the abattoir, 63 were from treated cattle and 67 were randomly selected using block randomization (every sixth sample; See Table S7) from the apparently healthy cattle (n=402).

2.2. Bacterial isolation

Isolation of *E. coli* was carried out following the method described by following B.FLT.3003. Briefly, ten (10) grams of faeces were added into 7 mL of sterile 0.1% buffered peptone water in a falcon tube. The mixture was vortexed and a sterile cotton tip applicator was used to seed it onto MacConkey agar and Brilliance ESBL agar (Thermofisher Scientific, Australia). A similar approach was used for faecal swab samples collected at the slaughter house. The sample was streaked using a sterile loop and incubated at 37°C ± 2°C for 24 hours. After incubation, one presumptive, well isolated colony was selected from the MacConkey agar and Brilliance ESBL agar, respectively. Similarly, to identify *Enterococcus* spp., the faecal mixture was plated and streaked onto Slanetz and Bartley agar plate (Thermofisher Scientific, Australia) following B.FLT.3003. The plate was incubated in to 37°C ± 2°C for 48 h. A single, well isolated red, maroon or pink coloured colony was carefully chosen, and subcultured onto sheep blood agar. Finally, the identity of all suspected target colonies was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonik GMBH, Germany) and stored in -80°C in tryptone soya broth with 20% glycerol.

1.3. Antimicrobial susceptibility testing

All isolates of *E. coli* and *Enterococcus* species were subjected to antimicrobial susceptibility testing. Commercially prepared plates were used to test the minimum inhibitory concentration of the isolates, following the Clinical and Laboratory Standards Institute and National Antimicrobial Resistance Monitoring System guidelines (CLSI, 2020; NARMS, 2011). For *E. coli*, phenotypic susceptibility was determined using the standard Sensititre NARMS Gram-negative CMV3AGNF MIC Plate that included amoxicillin-clavulanate, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (Table 1). The reference strains were *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. For *Enterococcus* spp., phenotypic susceptibility was determined using the Sensititre NARMS Gram-positive CMV3AGPF Plate that included chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamycin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin tartrate, and vancomycin (Table 2). The reference strains were *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213. To date, only a susceptible breakpoint has been established for tigecycline for enterococci. In this study, ≥ 0.5 µg/mL for tigecycline (NARMS) were used as the resistance cut-off values.

Table 1. Tested dilution ranges and breakpoints used for the antimicrobial susceptibility testing of *E. coli*.

Antimicrobial agent	Range	Breakpoints
Amoxicillin/clavulanic acid	1/0.5 - 32/16	≥ 32/16
Ampicillin	1 - 32	≥ 32
Azithromycin	0.12 - 16	> 16
Cefoxitin	0.5 - 32	≥ 32
Ceftiofur	0.12 - 8	≥ 8
Ceftriaxone	0.25 - 64	≥ 4
Chloramphenicol	2 - 32	≥ 32
Ciprofloxacin	0.015 - 4	≥ 1
Gentamycin	0.25 - 16	≥ 16
Nalidixic acid	0.5 - 32	≥ 32
Streptomycin	2 - 64	≥ 64
Sulfisoxazole	16 - 256	> 256
Tetracycline	4 - 32	≥ 16
Trimethoprim/ sulfamethoxazole	0.12/2.38 – 4/76	≥ 4/76

Table 2. Dilution ranges and breakpoints used for antimicrobial susceptibility testing of *Enterococcus* spp. isolates.

Antimicrobial agent	Range	Breakpoints
Chloramphenicol	2 - 32	≥ 32[a]
Ciprofloxacin	0.12 - 4	≥ 4[a]
Daptomycin	0.25 - 16	≥ 8[a]
Erythromycin	0.25 - 8	≥ 8[a]
Gentamicin	128 - 1024	≥ 512[b]
Kanamycin	128 - 1024	≥ 1024[b]
Lincomycin	1 - 8	≥ 8[b]
Linezolid	0.5 - 8	≥ 8[a]
Nitrofurantoin	2 - 64	> 64[a]
Penicillin	0.25 - 16	≥ 16[a]
Streptomycin	512 - 2048	≥ 1024[b]
Quinupristin/dalfopristin	0.5 - 32	≥ 4[a]
Tetracycline	1 - 32	≥ 16[a]
Tigecycline	0.015 – 0.5[c]	≥ 0.5[b]
Tylosin tartrate	0.25 - 32	≥ 32[b]
Vancomycin	0.25 - 32	≥ 32[a]

[a] Clinical and Laboratory Standards Institute guidelines; [b] National Antimicrobial Resistance Monitoring System; [c] only breakpoint for sensitivity established

2.4. Data analysis

The statistical software programs Excel (Microsoft Corp., Redmond, WA), STATA version 15.0 (Stata Corporation, College Station, TX, USA) and the R Statistical Package version 4.0.0 were used to process data for the bacterial isolates and analyse the AMR patterns of isolates for associations with relevant outcomes. Logistic regression models in STATA were used to evaluate the effect of treatment on the probability of bacteria being resistant to each antimicrobial drug tested. The odds ratio was used to assess the association between exposure to particular antimicrobial/s and the development of AMR. MDR was defined as resistance to at least three antimicrobial classes (Magiorakos et al., 2012). The effect of treatment on the AMR pattern was compared between treated and apparently healthy cattle. The frequency of resistance for each antimicrobial agent was described as rare: <0.1%; very low: 0.1% to 1.0%; low: >1.0% to 10.0%; moderate: >10.0% to 20.0%; high: >20.0% to 50.0%; very high: >50.0% to 70.0%; and extremely high: >70.0%; according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (EFSA)(EFSA, 2018).

3. Result

3.1. Antimicrobial resistance in *E. coli* isolates

3.1.1. The prevalence of AMR in E. coli isolated from clinically sick beef cattle

A total of 144 samples were collected from treated cattle, including 135 and 9 before and after the first treatment, respectively. Tulathromycin was the first treatment used in treated cattle. If the cattle individual did not recover, oxytetracycline was given a week later. In this study, *E. coli* was isolated from 90 (66.7%) of the rectal faecal samples collected from treated cattle before their first treatment. The antimicrobial susceptibility test showed the highest resistance to tetracycline (80.0%), followed by sulfisoxazole (17.8%), streptomycin (10.0%), ampicillin (5.6%), and azithromycin (5.6%). All isolates were sensitive to ciprofloxacin and gentamicin (Table 3).

A total of 48 (35.6%) ESBL-producing *E. coli* were isolated from treated cattle before receiving their first treatment. Absolute resistance to ampicillin, ceftiofur and ceftriaxone was observed, followed by tetracycline (93.8%), streptomycin (35.4%), and sulfisoxazole (35.4%). All ESBL-producing *E. coli* isolates were sensitive to gentamycin, amoxicillin/clavulanic acid, cefoxitin, chloramphenicol, ciprofloxacin and nalidixic acid (Table 4).

Table 3. The distribution of antimicrobial susceptibility testing results observed in *Escherichia coli* (n=90) isolated from treated cattle before their first treatment

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*														
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0	-					10.0	64.4	24.4	1.1								
	Streptomycin	10.0	8.97-11								3.3	50.0	35.6		1.1	3.3	6.7		
Beta lactam	Ampicillin	5.6	4.57-6.63							23.3	41.1	26.7	3.3			5.6			
	Amoxicillin/Clavulanic acid	1.1	0.07-2.13							8.9	40.0	40.0	10.0			1.1			
	Cefoxitin	1.1	0.07-2.13								4.4	52.2	40.0	2.2		1.1			
	Ceftiofur	2.2	1.17-3.23				4.4	52.2	41.1				1.1	1.1					
	Ceftriaxone	2.2	1.17-3.23					96.7	1.1				1.1				1.1		
Folate pathway inhibitor/antagonists	Sulfisoxazole	17.8	16.8-18.8											64.4	15.6	1.1	1.1		17.8
	Trimethoprim/Sulfamethoxazole	2.2	1.17-3.23				95.6	2.2					2.2						
Macrolides	Azithromycin	5.6	4.57-6.63							4.4	13.3	71.1	5.6		5.6				
Phenicols	Chloramphenicol	2.2	1.17-3.23								1.1	27.8	66.7	2.2		2.2			
Fluoroquinolones	Ciprofloxacin	0.0	-	95.6	3.3		1.1												
	Nalidixic acid	1.1	0.07-2.13							8.9	82.2	7.8				1.1			
Tetracycline	Tetracycline	80.0	79-81									16.7	3.3	4.4	6.7	68.9			

Table 4. The distribution of antimicrobial susceptibility testing results observed in ESBL-producing *Escherichia coli* (n=48) isolated from treated cattle before their first treatment

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*														
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0	-					18.8	64.6	16.7									
	Streptomycin	35.4	34.00-36.80									37.5	27.1			8.3	27.1		
Beta lactam	Ampicillin	100.0	98.60-100.00													100.0			
	Amoxicillin/Clavulanic acid	0.0	-									22.9	70.8	6.3					
	Cefoxitin	0.0	-									29.2	68.8	2.1					
	Ceftiofur	100.0	98.60-100.00										4.2	95.8					
	Ceftriaxone	100.0	98.60-100.00												6.3	37.5	56.3		
Folate pathway inhibitor/antagonists	Sulfisoxazole	35.4	34.00-36.80											18.8	20.8	25.0			35.4
	Trimethoprim/Sulfamethoxazole	33.3	31.90-34.70				62.5	2.1	2.1				33.3						
Macrolides	Azithromycin	22.9	21.50-24.30									41.7	35.4		22.9				
Phenicols	Chloramphenicol	0.0	-									20.8	75.0	4.2					
Fluoroquinolones	Ciprofloxacin	0.0	-	85.4				8.3	6.3										
	Nalidixic acid	0.0	-								72.9	18.8	8.3						
Tetracycline	Tetracycline	93.8	92.40-95.20									6.3			2.1	91.7			

In this study, 14 faecal samples were collected from treated cattle that did not recover after the tulathromycin treatment and were treated for a second course with oxytetracyline. Among these, *E. coli* was isolated in 9 (64.3%), while the ESBL-producing *E. coli* was isolated in 7 (50%). Only tetracycline (66.7%) and sulfisoxazole (11.1%) resistance were observed in *E. coli* isolated from cattle requiring second treatment (Table 5). In contrast, all ESBL-producing *E. coli* isolates were resistant to ampicillin, ceftiofur, ceftriaxone and tetracycline (Table 6). All isolates were sensitive to gentamicin, amoxicillin/clavulanic acid, cefoxitin, chloramphenicol, ciprofloxacin and nalidixic acid.

Table 5. Antimicrobial susceptibility test results from *Escherichia coli* (n = 9) isolated from treated cattle not recovered after the first treatment

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*															
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0	-					22.2	66.7	11.1									
	Streptomycin	0.0	-									66.7	33.3						
Beta lactam	Ampicillin	0.0	-							33.3	44.4	22.2							
	Amoxicillin/Clavulanic acid	0.0	-								66.7	33.3							
	Cefoxitin	0.0	-								11.1	55.6	33.3						
	Ceftiofur	0.0	-					55.6	44.4										
	Ceftriaxone	0.0	-					100.0											
Folate pathway inhibitor/antagonists	Sulfisoxazole	11.1	7.84-14.40											77.8	11.1				11.1
	Trimethoprim/Sulfamethoxazole	0.0	-				100.0												
Macrolides	Azithromycin	0.0	-								11.1	88.9							
Phenicols	Chloramphenicol	0.0	-									22.2	77.8						
Fluoroquinolones	Ciprofloxacin	0.0	-	100.0															
	Nalidixic acid	0.0	-							22.2	77.8								
Tetracycline	Tetracycline	66.7	63.40-69.90									33.3			22.2	44.4			

Table 6. Antimicrobial susceptibility test results from ESBL-producing *Escherichia coli* (n=7) isolated from treated cattle not recovered after the first treatment

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*															
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0	-					14.3	85.7										
	Streptomycin	28.6	24.90-32.30									57.1	14.3				28.6		
Beta lactam	Ampicillin	100.0	96.30-100.00													100.0			
	Amoxicillin/Clavulanic acid	0.0	-									28.6	71.4						
	Cefoxitin	0.0	-									28.6	71.4						
	Ceftiofur	100.0	96.30-100.00											100.0					
	Ceftriaxone	100.0	96.30-100.00													57.1	42.9		
Folate pathway inhibitor/antagonists	Sulfisoxazole	28.6	24.90-32.30											14.3	14.3	42.9			28.6
	Trimethoprim/Sulfamethoxazole	28.6	24.90-32.30				71.4						28.6						
Macrolides	Azithromycin	28.6	24.90-32.30									14.3	57.1		28.6				
Phenicols	Chloramphenicol	0.0	-									28.6	71.4						
Fluoroquinolones	Ciprofloxacin	0.0	-	100.0															
	Nalidixic acid	0.0	-								100.0								
Tetracycline	Tetracycline	100.0	96.30-100.00													100.0			

Of the 90 *E. coli* isolated from treated cattle, 72 (80.0%) were resistant to at least one of the tested antimicrobials. Among these, 46 (51.1%) were resistant to one antimicrobial class, 17 (18.9%) to two, 7 (7.8%) to three, and one of each with 1 (1.1%) isolate resistant to four and five antimicrobial classes respectively (Table 7). From these isolates, 9 (10.0%) were MDR. Sulfisoxazole and tetracycline resistance were the most commonly found combinations, with an overall occurrence of 16 (17.8%).

ESBL-producing *E. coli* isolates were isolated from 48 (35.6%) samples. All ESBL-producing *E. coli* isolates were resistant to at least one antimicrobial class. Of the ESBL-producing *E. coli* isolates, 18 (37.5%) were MDR. Ampicillin, ceftriaxone, ceftiofur and tetracycline were the most commonly observed antimicrobial resistance combinations, with a total prevalence of 93.7%. Nine (18.7%) ESBL-producing *E. coli* isolates were resistant to five antimicrobial classes, including aminoglycosides, betalactams, macrolides, sulfonamides and tetracyclines.

In addition, 14 samples were collected from treated cattle receiving second treatment. Of these, *E. coli* was identified from 9 samples and 5 (55.6%) of them were resistant to one antimicrobial class, whilst 3 (33.3%) isolates were sensitive to all antimicrobials tested. ESBL-producing *E. coli* was isolated from 7 (50.0%) of the samples and all isolates were resistant to all antimicrobials tested.

Table 7. The AMR pattern of *E. coli* isolated from treated cattle before their first and second treatment

	Non-ESBL(n=90)		ESBL (55)	
AMR pattern	**Before first treatment(n=90)**	**Before second treatment*(n=9)**	**Before first treatment (n=48)**	**Before second treatment* (n=7)**
All sensitive	18 (20.0)	3 (33.3)		
TET	46 (51.1)	5 (55.6)		
AMP-AXO-XNL			3 (6.2)	
AZI-TET	4 (4.4)			
FIS-TET	9 (10.0)	1 (11.1)		
AMP-AXO-TET-XNL	1 (1.1)		27 (56.2)	5 (71.4)
AMP-TET	1 (1.1)			
STR-TET	1 (1.1)			
NAL-TET	1 (1.1)			
AMP-STR-TET	1 (1.1)			
FIS-STR-TET	4 (4.4)			
FIS-TET-STR-SXT	1 (1.1)			
AMP-AXO-AZI-XNL-TET			1 (2.1)	
AMP-AUG-AXO-AZI-FOX-TET-XNL	1 (1.1)			
CHL-FIS-STR-TET	1 (1.1)			
AMP-AXO-FIS-STR-SXT-TET-XNL			7 (14.6)	
AMP-CHL-FIS-STR-TET-SXT	1 (1.1)			
AMP-AXO-AZI-FIS-STR-TET-XNL			1 (2.1)	
AMP-AXO-AZI-FIS-STR-SXT-TET-XNL			9 (18.7)	2 (28.6)
Non-MDR	63 (70.0)	6 (66.7)	30 (62.5)	5 (71.4)
MDR	9 (10.0)		18 (37.5)	2 (28.6)
Resistance	72 (80.0)	6 (66.7)	48 (100.0)	7 (100.0)

*****Draxxin treated**; AUG**, amoxicillin/clavulanic acid; **AMP**, ampicillin; **AZI**, azithromycin; **AXO**, ceftriaxone; **CHL**, chloramphenicol; **FIS**, sulfisoxazole; **FOX**, cefoxitin; GEN; **STR**, streptomycin; **TET**, tetracycline; **SXT**, trimethoprim/sulfamethoxazole; **XNL**, ceftiofur

3.1.2. The prevalence of AMR in *E. coli* isolated at the abattoir

A total of 56 (83.6%) *E. coli* were isolated from 67 rectal swab samples from apparently healthy cattle. The most common resistance was observed to tetracycline (79.0%), ampicillin (15.8%), sulfisoxazole (10.5%) and streptomycin (8.8%). A relatively lower resistance was detected to trimethoprim/sulfamethoxazole (3.5%) and chloramphenicol (1.8%) (Table 8). From the samples from apparently healthy cattle, only one ESBL-producing *E. coli* was isolated, and was resistant to ampicillin, azithromycin, ceftiofur, ceftriaxone, streptomycin, sulfisoxazole and tetracycline.

A total of 50 (79.4%) isolates of *E. coli* were isolated from 63 rectal swabs sampled from treated cattle. The resistance was observed to tetracycline (54.0%), sulfisoxazole (8.0%), ampicillin (8.0%), and streptomycin (4.0%) (Table 9). Of the samples from treated cattle, 4 (6.3%) ESBL-producing *E. coli* were isolated, and were resistant to ampicillin, ceftiofur, ceftriaxone and tetracycline.

Table 8. Antimicrobial susceptibility test results in *Escherichia coli* (n = 56) isolated from rectal swabs collected from apparently healthy cattle at the abattoir

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*															
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0						1.8	63.2	35.1									
	Streptomycin	8.8	7.46-10.10									50.9	33.3	5.3	1.8	3.5	5.3		
Beta lactam	Ampicillin	15.8	14.50-17.10							26.3	40.4	17.5				15.8			
	Amoxicillin/Clavulanic acid	0.0								5.4	50.0	40.4	5.3						
	Cefoxitin	0.0								1.8	33.3	33.3	31.6						
	Ceftiofur	0.0					22.8	54.4	22.8										
	Ceftriaxone	0.0						100.0											
Folate pathway inhibitor/antagonists	Sulfisoxazole	10.5	9.22-11.80											77.2	10.5	1.8			10.5
	Trimethoprim/Sulfamethoxazole	3.5	2.20-4.82				96.5						3.5						
Macrolides	Azithromycin	0.0									12.3	77.2	8.8		1.8				
Phenicols	Chloramphenicol	1.8	0.44-3.06								1.8	47.4	47.4	1.8		1.8			
Fluoroquinolones	Ciprofloxacin	0.0		98.3	1.8														
	Nalidixic acid	0.0								10.5	80.7	8.8							
Tetracycline	Tetracycline	79.0	77.90-80.30									21.1		8.8	31.6	38.6			

Table 9. Antimicrobial susceptibility test results in *Escherichia coli* (n = 50) isolated from rectal swabs collected from treated cattle at the abattoir

Antimicrobial class	Antimicrobial agent	Resistant (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)*															
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	0.0	-					6.0	76.0	16.0	2.0								
	Streptomycin	4.0	2.61-5.39									46.0	46.0	4.0		2.0	2.0		
Beta lactam	Ampicillin	8.0	6.61-9.39							28.0	46.0	18.0				8.0			
	Amoxicillin/Clavulanic acid	0.0	-							12.0	52.0	26.0	10.0						
	Cefoxitin	0.0	-							2.0	32.0	42.0	22.0	2.0					
	Ceftiofur	0.0	-				20.0	50.0	30.0										
	Ceftriaxone	0.0	-					100.0											
Folate pathway inhibitor/antagonists	Sulfisoxazole	8.0	6.61-9.39											76.0	12.0	2.0	2.0		8.0
	Trimethoprim/Sulfamethoxazole	0.0	-				100.0												
Macrolides	Azithromycin	0.0	-					2.0			24.0	72.0	2.0						
Phenicols	Chloramphenicol	0.0	-								2.0	40.0	58.0						
Fluoroquinolones	Ciprofloxacin	0.0	-	98.0	2.0														
	Nalidixic acid	0.0	-						2.0	10.0	86.0	2.0							
Tetracycline	Tetracycline	54.0	52.60-55.40									44.0	2.0	8.0	22.0	24.0			

Cattle were given metaphylactic and / or therapeutic treatments based on their origin and health condition. Tilmicosin (Bovatil) and in-feed Chlorotetracycline (CTC200) were the most commonly used metaphylactic treatments, while Oxytetracycline (Bivatop200) and Tulathromycin (Draxxin) were used in treatment cattle. Based on the type of treatment they received during the feedlot phase, all cattle were grouped into six categories. In total, *E. coli* was isolated from 14 CTC200 (13.2%), 42 CTC200+Bovatil (39.6%), 27 CTC200+Draxxin (25.5%), 2 Draxxin+Bivatop200 (1.9%), 10 CTC200+Bovatil+Draxxin (9.4%) and 11 CTC200+Draxxin+Bivatop200 (10.4%) exposed cattle that were sampled. In *E. coli* isolated from CTC200 exposed cattle, resistance was observed to tetracycline (85.7%), sulfisoxazole (14.3%) and streptomycin (14.3%) (Table S1). A slightly lower resistance was detected in *E. coli* isolated from CTC200+Bovatil exposed cattle. Overall lower resistance levels were detected in *E. coli* isolated from treated cattle receiving therapeutic treatment (Figure1).

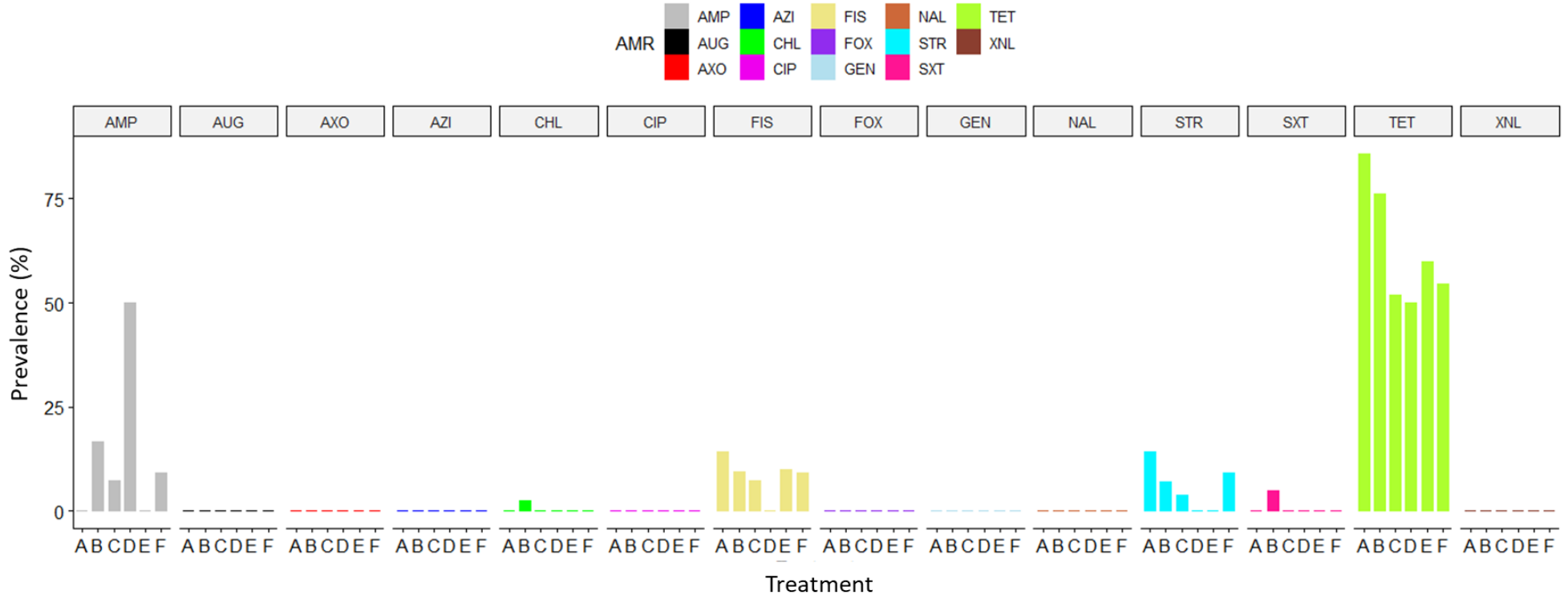

Figure 1. Analysis of the antimicrobial resistance prevalance (% of isolates) in *Escherichia coli* isolated from rectal faeces collected at the abattior (n=106). The type of antimicrobial cattle were exposed to includes; **A**= CTC200 (n=14), **B**= CTC200+Bovatil (n=42), **C** = CTC200+Draxxin (n=27), **D**= Draxxin+Bivatop200 (n=2), **E**=CTC200+Bovatil+Draxxin (n=10), **F**=CTC200+Draxxin+Bivatop200 (n=11). The resistance outcomes are displayed for each antimicrobial**; AUG** (Amoxicillin/clavulanic acid), **AMP** (Ampicillin), **AXO** (Ceftriaxone), **AZI** (Azithromycin), **CHL** (Chloramphenicol), **CIP**(Ciprofloxacin), **FIS** (Sulfisoxazole), **FOX** (Cefoxitin), **GEN** (Gentamcin), **NAL** (Nalidixic acid), **STR** (Streptomycin), **SXT** (Trimethoprim/sulfamethoxazole), **TET** (Tetracycline) and **XNL** (Ceftiofur).

The highest AMR was observed in *E. coli* isolated from cattle exposed only to metaphylaxis. *E. coli* isolated from cattle that received CTC200 alone and CTC200+Bovatil showed the highest AMR, with a prevalence rate of 85.7% and 76.2%, respectively. The highest number of AMR pattern and MDR was detected in *E. coli* isolated from CTC200 +Bovatil exposed cattle (Table 10).

Table 10. The antimicrobial resistance pattern of *E. coli* isolated from reactal swabs collected at the abattior

	Type of antimicrobial used (Number of cattle)					
AMR pattern	**CTC200 (14)**	**CTC200+Bovatil (42)**	**CTC200+Draxxin (27)**	**Draxxin+Bivatop200 (2)**	**CTC200+Bovatil+ Draxxin (10)**	**CTC200+Draxxin+ Bivatop200 (11)**
All sensitive	2 (14.3)	10 (23.8)	13 (48.1)	1 (50.0)	4 (40.0)	5 (45.4)
TET	8 (57.1)	21 (50.0)	10 (37.0)		5 (50.0)	4 (36.4)
AMP-TET	2 (14.3)	5 (11.9)	2 (7.4)	1 (50.0)		2 (18.2)
FIS-TET		1 (2.4)	1 (3.7)		1 (10.0)	
FIS-TET-SXT		2 (4.8)				
AMP-STR-TET		2 (4.8)				
FIS-STR-TET	2 (14.3)		1 (3.7)			1 (9.1)
CHL-FIS-STR-TET		1 (2.4)				
Non-MDR	10 (71.4)	29 (69.0)	13 (48.1)	1 (50.0)	6 (60.0)	6 (54.5)
MDR	2 (14.3)	3 (7.1)	1 (3.7)			1 (9.1)
Resistance	12 (85.7)	32 (76.2)	14 (51.8)	1 (50.0)	6 (60.0)	7 (63.6)

AMP (Ampicillin), **CHL** (Chloramphenicol), **FIS** (Sulfisoxazole), **STR** (Streptomycin), **SXT** (Trimethoprim/sulfamethoxazole), **TET** (Tetracycline)

The AMR levels differed in *E. coli* isolated from cattle that were exposed to different antimicrobials. In this study, CTC200, CTC200 + Bovatil, CTC200 + Bovatil + Draxxin, CTC200 + Draxxin + Bivatop200 increased the AMR in *E. coli* by 5.1, 2.8, 1.5 and 1.4 times compared to unexposed cattle, respectively without overall significance (P>0.05; Table 11).

Table 11. The association of different treatment on the development of AMR in *E. coli*

Antimicrobial treatment	Odds Ratio	p-value	[95% CI]
CTC200	5.085	0.371	0.14-179.53
CTC200+Bovatil	2.797	0.543	0.10-76.66
CTC200+Draxxin	0.978	0.989	0.03-27.01
Draxxin+Bivatop200	0.718	0.844	0.03-19.51
CTC200+Bovatil+Draxxin	1.483	0.823	0.05-47.18
CTC200+Draxxin+Bivatop200	1.392	0.844	0.05-37.81

3.2. Antimicrobial resistance in *Enterococcus* isolates

3.2.1. The prevalence of AMR in *Enterococcus* spp. isolated from sick cattle

Overall, 7 species of enterococci were isolated from treated cattle before receiving their first treatment and their relative abundance, in order of frequency included *E. faecium* (n=45), *E. hirae* (n=12), *E. durans* (n=11), E. *mundtti* (n=4), *E. raffinosus*, and *E. thailandicus* and *E. villorum* (n=1 each). High lincomycin resistance was observed in all species, with the prevalence rate of 53.3%, 100.0% and 88.9% in *E. faecium*, *E. hirae,* and the other group of species, respectively. In *E. faecium*, the highest resistance was observed to tetracycline (71.1%) followed by lincomycin (53.3%), ciprofloxacin (35.6%), erythromycin (26.7%), tylosin (24.4%), quinupristin /dalfopristin (17.8%) and daptomycin (15.6%). In *E. hirae*, all isolates were resistant to lincomycin, followed by tetracycline (75.0%), daptomycin, erythromycin, and tylosin at 16.7% each. The highest prevalence of nitrofurantoin and tigecycline resistance was observed in the other group of enterococci. Nitrofurantoin and tigecycline resistance were found only in one of each *E. thailandicus* and *E. durans* isolates. All isolates were sensitive to gentamycin, linezolid, penicillin and vancomycin (Table S2).

A total of 14 samples were collected from cattle that not recovered after the first treatment. Among these, four different types of enterococci were identified, namely *E. faecium* (n=3), *E. hirae* (n=2)*, E. durans* (n=2)*,* and *E. mundtti* (n=1). Overall, the highest prevalence of AMR was detected to lincomycin (87.5%), tetracycline (75.0%), and erythromycin and tylosin, 37.5% each. Some 12.5% of the isolates were resistant to daptomycin and quinupristin /dalfopristin (Table 12).

Table 12. The antimicrobial susceptibility testing in *Enterococcus* (n=8) isolated from treated cattle not recovered after the first treatment

Antimicrobial class	Antimicrobial agent	Resistance (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)																
				0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Aminoglycosides	Gentamycin	0.0	-														100				
	Kanamycin	0.0	-														87.5	12.5			
	Streptomycin	0.0	-																100.0		
Beta lactam	Pencillin	0.0	-					12.5	37.5	25.00	12.5	12.5									
Fluoroquinolones	Ciprofloxacin	0.0	-					12.5	37.5	25.0	25.0										
Glycopeptides	Vancomycin	0.0	-						87.5	12.5											
Glycylcyclines	Tigecycline	0.0	-				62.5	37.5													
Lincosamide	Lincomycin	87.5	84.00-91.00							12.5				87.5							
Lipopeptides	Daptomycin	12.5	9.04-16.00								50.0	37.5	12.5								
Macrolides	Erythromycin	37.5	34.00-41.00					37.5	12.5			12.5		37.5							
	Tylosine tartrate	37.5	34.00-41.00							25	37.5					37.5					
Nitrofurantoins	Nitrofurantoin	0.0	-												37.5	62.5					
Oxazolidinones	Linezolid	0.0	-							62.5	37.5										
Phenicols	Chloramphenicol	0.0	-									87.5	12.5								
Streptogramins	Quinupristin /dalfopristin	12.5	9.04-16.00						12.5	12.5	62.5				12.5						
Tetracycline	Tetracycline	75.0	71.50-78.50							25.0						75.0					

The AMR pattern of *Enterococcus* species isolated from treated cattle before and after treatment is shown in Table 13. Overall, *E. faecium* was the most frequently isolated species from treated cattle before treatment. The isolates were resistant to one (22.2%), two (31.1%), three (15.6%), four (17.8%), and five (6.7%) antimicrobial classes, with 18 isolates (40.0%) identified as MDR. The *E. faecium* isolates from cattle that received the first treatment were resistant to one, three, and four antimicrobial classes. However, all *E. hirae* were resistant to at least one antimicrobial class. Of the 12 *E. hirae* isolates, 2 (16.7%) were resistant to one antimicrobial, 7 (58.3%) to two, 3 (25.0%) to three antimicrobials classes. In total, three isolates (25.0%) were MDR. The MDR of other group of Enterococcus species were 3 (16.7%), whilst just two (11.1%) were sensitive to all tested antimicrobials.

Table 13. The antimicrobial resistance pattern of enterococci (n=75) isolated from treated cattle before the first and second treatment

AMR pattern	Enterococcus species					
	E. faecium		*E. hirae*		Others	
	Before first treatment (n=45)	Before second treatment(n=3)*	Before first treatment (n=12)	Before second treatment (n=2)*	Before first treatment (n=18)	Before second treatment (n=3)*
All sensitive	3 (6.7)				2 (11.1)	
CIP	3 (6.7)					
Dap	1 (2.2)					
LIN	1 (2.2)		2 (16.7)		5 (27.8)	1 (33.3)
TET	5 (11.1)	1 (33.3)				
CHL-TET	3 (6.7)					
CIP-TET	1 (2.2)					
CIP-NIT	1 (2.2)					
DAP-LIN	4 (8.9)		1 (8.3)	1 (50.0)		
ERY-TET	1 (2.2)					
LIN-NIT					1 (5.6)	
LIN-TET	4 (8.9)		6 (50.0)		7 (38.9)	2 (66.7)
CIP-Q/D-TET	1 (2.2)					
CIP-LIN-TET	1 (2.2)					
DAP-LIN-TET			1 (8.3			
LIN-Q/D-TET	2 (4.4)				1 (5.6)	
LIN-TET-TIG					1 (5.6)	
ERY-LIN-TET-TYL	3 (6.7)	1 (33.3)	2 (16.7)	1 (50.0)	1 (5.6)	
CIP-LIN-Q/D-TET	2 (4.4)					
DAP-LIN-Q/D-TET	1 (2.2)					
CIP-ERY-Q/D-TET-TYL	2 (4.4)					
CIP-ERY-LIN-TET-TYL	3 (6.7)					
ERY-LIN-Q/D-TET-TYL		1 (33.3)				
ERY-KAN-LIN-STR-TET-TYL	1 (2.2)					
CIP-ERY-LIN-NIT-TET-TYL	1 (2.2)					
CIP-DAP-ERY-LIN-TET-TYL	1 (2.2)					
Non-MDR	24 (53.3)	1 (33.3)	9 (75.0)	1 (50.0)	13 (72.2)	3 (100.0)
MDR	18 (40.0)	2 (66.7)	3 (25.0)	1 (50.0)	3 (16.7)	
Resistance	42 (93.3)	3 (100.0)	12 (100.0)	2 (100.0)	16 (88.9)	3 (100.0)

*Draxxin treated, **CHL** (Chloramphenicol), **CIP** (Ciprofloxacin), **DAP** (Daptomycin), **ERY** (Erythromycin), **KAN** (Kanamycin), **LIN** (Lincomycin), **NIT**(Nitrofurantoin), **Q/D** (Quinupristin/dalfopristin), **STR** (Streptomycin), **TET** (Tetracycline), **TIG** (Tigecycline), **TYL** (Tylosin tartrate)

3.2.2. *Enterococcus* isolated from abattoir

The isolation rate of *Enterococcus* spp from rectal swabs from apparently healthy cattle is presented in Table S3. *Enterococcus* was isolated from 55 (82.1%) of 67 samples, and the most commonly identified species were *E. hirae* (n=36), *E. faecium* (n=14), *E. durans* (n=3), and *E. mundtti* (n=2). *E. hirae* isolates were resistant to more antimicrobial classes compared to others, including exhibiting resistance to tetracycline (50.0%), daptomycin (27.8%), tylosin (27.8%) and erythromycin (19.4%). In *E. faecium*, the highest resistance was observed to lincomycin (78.6%), nitrofurantoin (50.0%), tigecycline, and ciprofloxacin (14.3% each). All isolates were sensitive to chloramphenicol, gentamycin, kanamycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin and vancomycin.

Among the enterococci isolated from treated cattle (n=63), *E. hirae* (n=37) was the most abundant species followed by *E. faecium* (n=11), *E. durans (n=2),* and *E. sulfureus* and *E. thailandicus* (1 of each). In *E. hirae* isolates, the highest resistance was observed to lincomycin (86.5%), followed by tetracycline (40.5%), tylosin (35.1%), daptomycin, and erythromycin (29.7% each). In *E. faecium* isolates, a slightly lower resistance to lincomycin (63.6%) and daptomycin (18.2%) was detected. Quinupristin/dalfopristin resistance was observed at 27.3% of the isolates. In addition, only one isolate was resistant to ciprofloxacin, erythromycin, nitrofurantoin, tetracycline, tigecycline, and tylosin (Table S4).

There were obvious differences in the relative abundance of enterococci among sample types, with some species clearly predominant in certain environments and sampling points. *E. faecium* was the most prevalent species from treated cattle and *E. hirae* from the abattior.

Assessment of the impact of antimicrobial use on the development of AMR E. hirae

The highest resistance observed in this study in *E. hirae* was to daptomycin, erythromycin, lincomycin, tetracycline, and tylosin (Table S5). Lincomycin resistance was observed in 100.0, 90.9, 90.0, 71.4 and 90.0% in *E. hirae* isolated from cattle that were exposed to CTC200, CTC200+Bovatil, CTC200+Draxxin, CTC200+Bovatil+Draxxin and CTC200+Draxxin+Bivatop200 respectively (Figure 2). The effect of the exposure to particular antimicrobials on AMR development was not significant.

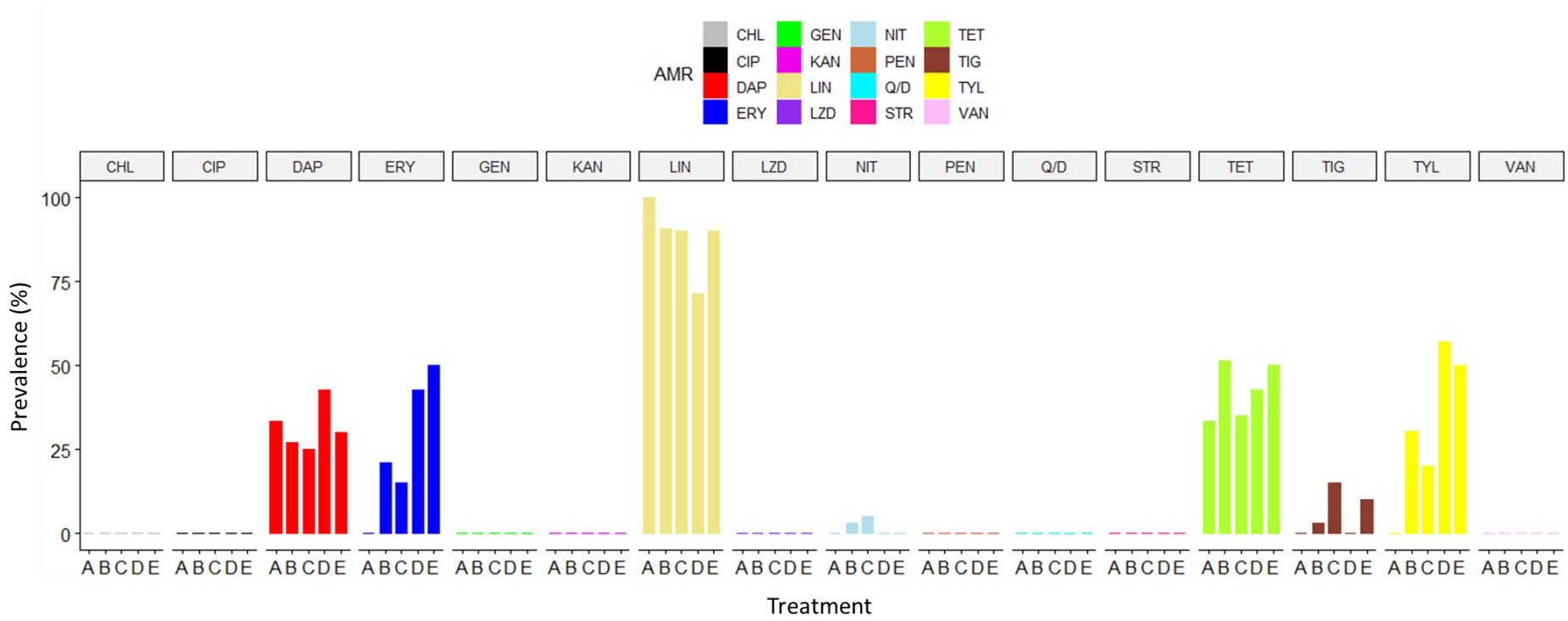

Figure 2. The prevalence of antimicrobial resistance in *Enterococcus hirae* isolated from rectal faeces collected from the abattior (n=73). The type of antimicrobial cattle were exposed to includes; **A**= CTC200(n=3), **B**= CTC200+Bovatil (n=33), **C** = CTC200+Draxxin (n=20), **D**=CTC200+Bovatil+Draxxin (n=7), **E**=CTC200+Draxxin+Bivatop200 (n=10). The resistance outcomes are displayed for each antimicrobial**; CHL** (Chloramphenicol), **CIP** (Ciprofloxacin), **DAP** (Daptomycin), **ERY** (Erythromycin), **GEN** (Gentamycin), **KAN** (Kanamycin) **LIN** (Lincomycin), **LZD** (Linezolid), **NIT** (Nitrofurantoin), **PEN** (Penicillin), **Q/D** (Quinupristin/dalfopristin), **STR** (Streptomycin), **TET** (Tetracycline), **TIG** (Tigecycline), **TYL** (Tylosin tartrate), **VAN** (Vancomycin)

The resistance pattern of *E. hirae* isolated from cattle that were exposed to different antimicrobials is shown in Table 14. Overall, 100.0, 95.0, 93.9, 90.0 and 71.4% resistant isolates of *E. hirae* were isolated from cattle that received CTC200, CTC200+Draxxin, CTC200+Bovatil, CTC200+Draxxin+Bivatop200 and CTC200+Bovatil+Draxxin respectively. The isolates with highest AMR pattern were detected from cattle that received CTC200+Bovatil and CTC200+Draxxin treatment. The proportions of MDR *E. hirae* were 57.1% and 50.0% in CTC200+Bovatil+Draxxin and CTC200+Draxxin+Bivatop200-treated cattle, respectively.

Table 14. The antimicrobial resistance pattern of *Enterococcus hirae* (n=73) isolated from cattle exposed to different antimicrobials

	Type of antimicrobials used				
AMR pattern	**CTC200 (3)**	**CTC200+Bovatil (33)**	**CTC200+Draxxin (20)**	**CTC200+Bovatil+ Draxxin (7)**	**CTC200+Draxxin+ Bivatop200 (10)**
All sensitive		2 (6.1)	1 (5.0)	2 (28.6)	1 (10.0)
DAP			1 (5.0)		
LIN	2 (66.7)	9 (27.3)	7 (35.0)		3 (30.0)
TET		1 (3.0)			
DAP-LIN		1 (3.0)		1 (14.3)	1 (10.0)
LIN-NIT		1 (3.0)	1 (5.0)		
LIN-TET		6 (18.2)	2 (10.0)		
LIN-TIG			2 (10.0)		
LIN-TYL		3 (9.1)	1 (5.0)		
DAP-LIN-TET	1 (33.3)	3 (9.1)	2 (10.0)		
DAP-LIN-TYL				1 (14.3)	
ERY-LIN-TET-TYL		2 (6.1)	1 (5.0)	2 (28.6)	2 (20.0)
DAP-ERY-LIN-TET-TYL		4 (12.1)	1 (5.0)	1 (14.3)	2 (20.0)
ERY-LIN-TET-TIG-TYL					1 (10.0)
DAP-ERY-LIN-TET-TIG-TYL		1 (3.0)	1 (5.0)		
Non-MDR	2 (66.7)	21 (63.6)	14 (70.0)	1 (14.3)	4 (40.0)
MDR	1 (33.3)	10 (30.3)	5 (25.0)	4 (57.1)	5 (50.0)
Resistance	3 (100.0)	31 (93.9)	19 (95.0)	5 (71.4)	9 (90.0)

DAP (Daptomycin), **ERY** (Erythromycin), **LIN** (Lincomycin), **NIT** (Nitrofurantoin), **TET** (Tetracycline), **TIG** (Tigecycline), **TYL** (Tylosin tartrate)

Assessment of the impact of antimicrobial use on the development of AMR E. faecium

Ciprofloxacin and quinupristin/dalfopristin resistance were detected in *E. faecium* sampled from the abattoir (Figure 3). One isolate (50.0%) from CTC200+Bovatil+Draxxin exposed cattle was resistant to ciprofloxacin and tetracycline (Table S6). Three isolates were recovered from cattle that were exposed to CTC200+Draxxin+Bivatop200. Of these, only one isolate (33.3%) was resistant to quinupristin/dalfopristin and tylosin. Isolates from cattle exposed to CTC200 (n=8) and CTC200 + Draxxin + Bivatop200 (n=3) were 100% resistant to lincomycin. There was no statistical difference in the effects of the exposure to particular antimicrobials on the AMR development.

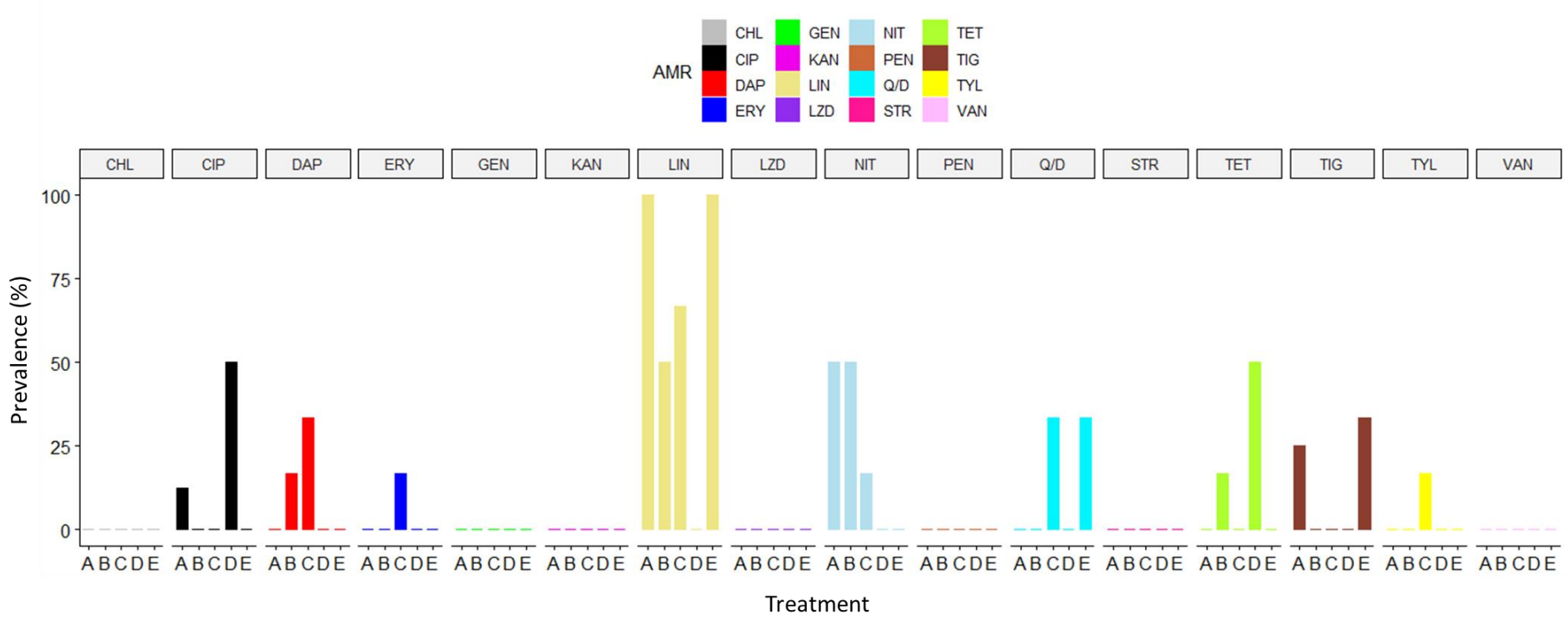

Figure 3. The prevalence of antimicrobial resistance in *Enterococcus faecium* isolated from rectal faeces collected from the abattior (n=25). The type of antimicrobial cattle were exposed to includes; **A**= CTC200 (n=8), **B**= CTC200+Bovatil (n=6), **C** = CTC200+Draxxin (n=6), **D**=CTC200+Bovatil+Draxxin (n=2), **E**=CTC200+Draxxin+Bivatop200 (n=3). The resistance outcomes are displayed for each antimicrobia**;** **CHL** (Chloramphenicol), **CIP** (Ciprofloxacin), **DAP** (Daptomycin), **ERY** (Erythromycin), **GEN** (Gentamycin), **KAN** (Kanamycin) **LIN** (Lincomycin), **LZD** (Linezolid), **NIT** (Nitrofurantoin), **PEN** (Penicillin), **Q/D** (Quinupristin/dalfopristin), **STR** (Streptomycin), **TET** (Tetracycline), **TIG** (Tigecycline), **TYL** (Tylosin tartrate), **VAN** (Vancomycin)

All *E. faecium* isolates from cattle exposed to CTC200, CTC200+Bovatil, CTC200+Draxxin, and CTC200+Draxxin+Bivatop200 showed absolute resistance (Table 15). A single isolate from CTC200+Bovatil+Draxxin treated cattle was sensitive to all tested antimicrobials. A total of 2 (25.0%), 1 (16.7%) and 1 (33.3%) of isolates from CTC200, CTC200 + Draxxin, CTC200 + Draxxin + Biivatop200 exposed cattle, respectively were MDR. The use of CTC200+Draxxin and CTC200+Bovatil increased the level of resistance by 4.3 and 3.4 times compared to non-exposed cattle, respectively with no significance in the differences (Table 19).

Table 15: The antimicrobial resistance pattern of *Enterococcus* faecium (n=25) isolated from cattle that were exposed to different antimicrobials

	Type of antimicrobials used				
AMR pattern	**CTC200 (8)**	**CTC200+Bovatil (6)**	**CTC200+Draxxin (6)**	**CTC200+Bovatil+ Draxxin (2)**	**CTC200+Draxxin+ Bivatop200 (3)**
All sensitive				1 (50.0)	
DAP			1 (16.7)		
LIN	3 (37.5)	1 (16.7)	2 (33.3)		2 (66.7)
NIT		1 (16.7)			
TET		1 (16.7)			
CIP-LIN	1 (12.5)				
CIP-NIT		1 (16.7)			
CIP-TET				1 (50.0)	
DAP-LIN		1 (16.7)			
LIN-NIT	2 (25.0)	1 (16.7)			
LIN-Q/D			1 (16.7)		
ERY-Q/D-TYL			1 (16.7)		
DAP-LIN-NIT			1 (16.7)		
LIN-NIT-TIG	2 (25.0)				
LIN-Q/D-TIG					1 (33.3)
Non-MDR	6 (75.0)	6 (100.0)	5 (83.3)	1 (50.0)	2 (66.7)
MDR	2 (25.0)	-	1 (16.7)	-	1 (33.3)
Resistance	8 (100.0)	6 (100.0)	6 (100.0)	1 (50.0)	3 (100.0)

CIP (Ciprofloxacin), **DAP** (Daptomycin), **ERY** (Erythromycin), **LIN** (Lincomycin), **NIT** (Nitrofurantoin), **Q/D** (Quinupristin/dalfopristin), **TET** (Tetracycline), **TIG** (Tigecycline), **TYL** (Tylosin tartrate)

Table 16. The association of different exposure to antimicrobials on the development of AMR in enterococcci

Antimicrobial treatment	Odds Ratio	p-value	[95% CI]
CTC200	1	.	.
CTC200+Bovatil	3.417	0.243	0.434-26.88
CTC200+Draxxin	4.333	0.25	0.357-52.58
CTC200+Bovatil+Draxxin	0.444	0.427	0.06-3.28
CTC200+Draxxin+Bivatop200	1	.	.

4. Discussion

The aim of this study was to determine if antimicrobial use in the feedlot contributed to the AMR of indicator commensal bacteria, *E. coli* and enterococci. In this study, *E. coli* were isolated from rectal faeces / swabs collected from treated and apparently healthy cattle. The highest resistance at slaughter was observed to tetracycline (54.0%), sulfisoxazole (8.0%), ampicillin (8.0%), and streptomycin (4.0%) in *E. coli* isolated from treated cattle. However, the prevalence of AMR in *E. coli* isolated from apparently healthy cattle was higher than in treated cattle. The most common resistances observed in *E. coli* isolated from apparently healthy cattle were to tetracycline (79.0%), ampicillin (15.8%), sulfisoxazole (10.5%) and streptomycin (8.8%). It should, be noted, that the vast majority of apprantely healthy cattle recevied meta-phylaxis with chlorotetracyline and/or tilmicosin at entry into the feedlot. A lower resistance was reported in B.FLT.3003 report, in which *E. coli* isolated at the abattoir was resistant to tetracycline (17.8%), ampicillin (5.4%), streptomycin (4.6%), and sulfisoxazole (3.9%). The difference in the prevalence could be due to changes in the source of the study animals, levels and types of antimicrobials used, nutrition, and seasonal variations. Antimicrobials are used in confined food animal production to prevent and treat different bacterial diseases. They benefit the health and well-being of food animals, while potentially pose risks due to the selection for resistant microorganisms. The use of antimicrobials has been associated with the risk of development of antimicrobial resistance (AMR) in key microorganisms including *E. coli* and *E. faecium.* In this study, Draxxin, Bivatop200 and Excede were the antimicrobials used in treated cattle. In addition, tetracycline-based product (CTC200) and a macrolide-based product (Bovatil) were used for the metaphylaxis of cattle coming from high-risk areas.

In this study, the major resistance in *E. coli* could be associated with the use of tetracycline-based metaphylaxis. Furthermore, the AMR bacteria could be acquired from the environment, particularly for antimicrobials that were not used at all such as streptomycin and sulfisoxazole. In particular, the use of chlorotetracycline had raised concerns beyond resistance to tetracycline, as AMR to other antimicrobial classes could occur. The occurrence of AMR towards other antimicrobial classes associated with chlortetracycline (CTC) use occurs due to variety of mobile genetic elements with multiple antimicrobial resistance genes stimulated by the use of CTC, each of which gives resistance to another antimictrobial class (Durso and Cook, 2014a). A study in one of the United States beef cattle showed that administration of CTC to prevent liver abscesses in beef cattle increased tetracycline resistance genes such as *tetA, tetB* and *tetM* in faeces (Vikram et al., 2017). The tetracycline resistance genes encode ribosomal protection proteins, and among these genes, *tetM*, *tetQ*, *tetS*, and *tetW* are

sometimes found in conjugative transposons, implying a high potential for genetic exchange in some bacteria (Lancaster et al., 2004; Melville et al., 2004).

Escherichia coli are significant producers of Extended Spectrum ß-Lactamases (ESBLs) and are increasing in livestock production (Smet et al., 2010). In this study, the prevalence of ESBL-producing *E. coli* from treated cattle before receiving their first treatment was 48 (35.6%). It is important to note that the rectal samples from treated cattle were collected before their treatment. Hence, ESBL-producing strains of *E. coli* were present without exposure to antimicrobials. We hypothesise that in treated cattle, illness has resulted in a shift of the gut microbiota favouring AMR strains. Additionally, the prevalence of ESBL-producing *E. coli* from treated cattle was 4 (6.3%), with only one isolate (1.5%) detected from apparently healthy cattle from rectal swabs collected at the abattior. All ESBL-producing isolates were resistant to ampicillin, ceftiofur, ceftriaxone and tetracycline.

Of the 63 enterococci. isolated from treated cattle, only 11 were *E. faecium* being an important human pathogen. In these isolates, the highest resistance was detected to lincomycin (63.6%) followed by quinupristin/dalfopristin (27.3%) and daptomycin (18.2%). In contrast, 14 *E. faecium* isolates were recovered from apparently healthy cattle and were resistant to lincomycin (78.6%), nitrofurantoin (50.0%), tigecycline, and ciprofloxacin (14.3% each). This result coincided with B.FLT.3003 report, in which *E. faecium* isolated from the rectal swabs collected at the abattoir was resistant to lincomycin (82.9%), followed by nitrofurantoin (61.5%), quinupristin/dalfopristin (21.4%), daptomycin (17.9%) and ciprofloxacin (9.4%). The resistance in nitrofurantoin mainly occurs due to mutations in *nfsA* and/or *nfsB*, both of which encode oxygen insensitive nitroreductases (Shakti and Veeraraghavan, 2015). In addition, the plasmid-mediated efflux genes, *oqxAB,* are associated with high levels of nitrofurantoin resistance (Ho et al., 2016). However, neither mutation nor resistance gene were detected against nitrofurantoin in B.FLT.3003. Therefore, the mechanism of nitrofurantoin resistance in beef cattle may not be fully understood and further work is required to be explained. Similarly, daptomycin resistance is reported to be linked with mutations of genes encoding the cell envelope stress response (*LiaFSR* and *YycFGHIJ*) and the genes responsible in the metabolism of phospholipids (*gdpD* and *cls*) (Arias et al., 2011; Bender et al., 2018). In B.FLT.3003, the WGS analysis showed no mutation on the target genes. It is likely the resistance is not yet fully elicited and this is an area that requires further work. Finally, resistance to quinupristin/dalfopristin in B.FLT.3003 did not find resistance genes and the mechanism is yet to be elicited. However, after this study was completed, some whole genome sequencing detected *vat (E)* responsible for virginiamycin and Q/D resistance in one and *erm (B)* responsible for erythromycin resistance but may also cross resist with streptogramin

B in three isolates. It is worth noting that no streptogramin antimicrobials have been used on this feedlot for over two years.

In this study, cattle exposed to metaphylaxis showed a trend for higher levels of resistance (without significance) for any of the antimicrobial / bacteria combinations. This finding may be true or, more likely, confounded by the small number of samples collected for each combination, often disproportionate, and using information from a single feedlot. Indeed, this would be expected even in a larger field study as most products used for metaphylaxis are prescribed at low administration doses (i.e. subtherapeutic dose), being previously highly correlated with adverse effects on the sensitive bacterial populations (Fauci and Marston l, 2014; Pokharel et al., 2020; Tangcharoensathien et al., 2018). In other words, exposure to subtherapeutic doses stimulates development of AMR and a shift in distribution of sensitive and resistant strains.

5. Conclusions

This study estimated the effects of exposure to antimicrobial/s on the emergence of AMR in *E. coli* and enterococci. The highest resistance in *E. coli* isolates was to tetracycline in both treated and apparently healthy cattle. This could be due to the widespread use of tetracycline for metaphylaxis and therapeutic purposes on the farm. There were also obvious differences in the relative abundance of enterococci among sample types, with some species clearly predominant in certain environments and sampling points. *E. faecium* was the most prevalent species from treated cattle at the feedlot and *E. hirae* from the abattior. Metaphylaxis could be one of the main driving forces for the emergence of AMR bacteria in beef feedlots, however further work is required as only limited number of cattle from non-metaphylaxis pens were sampled.

This study observed that some *E. coli* isolates were resistant to critically important antimicrobials such as ceftiofur and ceftriaxone. Although majority of ESBL-producing isolates originated from treated cattle (NOTE: before treatment), the prevalence of AMR bacteria may not be directly correlated with the use of antimicrobials, and even cattle without antimicrobial treatment contained ESBL-producing *E. coli*. The results from this study would indicate that further research areas or recommendations to the industry include

1. Continuous surveillance of AMR in feedlot indicator bacteria is essential, both pre-treatment and at slaughter
2. Of particular importance are
    a. The 3rd generation cephalosporin resistance in *E. coli* isolated from cattle pre-treatment that decreases over time to low prevalence by slaughter
    b. Daptomycin, Quinopristin / Dalfopristin and Nutrofurantoin resistances in enterococci, including genetic mechanisms of their resistance
3. Whole-genome sequence of all resistant and representative control isolates from this project is essential to
    a. Compare them with the pig, poultry, and human clinical isolates from Australia
    b. Potentially detect novel mechanisms of resistance in isolates with high disproportion between genotypic (detected by whole genome sequencing) and phenotypic resistance (already established during this project)
4. Studies of how antimicrobial resistance is acquired from the environment
5. Larger sample size from multiple feedlots and pens should be included in the future research
6. The industry should continue to focus on implementation of the antimicrobial stewardship guidelines
7. The use of metaphylaxis in feedlot industry should be minimised

6. References

Abreham, S., Teklu, A., Cox, E., and Sisay Tessema, T. (2019). Escherichia coli O157:H7: distribution, molecular characterization, antimicrobial resistance patterns and source of contamination of sheep and goat carcasses at an export abattoir, Mojdo, Ethiopia. *BMC Microbiol* **19**, 215.

Arias, C. A., Panesso, D., McGrath, D. M., Qin, X., Mojica, M. F., Miller, C., Diaz, L., Tran, T. T., Rincon, S., Barbu, E. M., Reyes, J., Roh, J. H., Lobos, E., Sodergren, E., Pasqualini, R., Arap, W., Quinn, J. P., Shamoo, Y., Murray, B. E., and Weinstock, G. M. (2011). Genetic Basis for In Vivo Daptomycin Resistance in Enterococci. *New England Journal of Medicine* **365**, 892-900.

Badger, S., Sullivan, K., Jordan, D., Caraguel, C., Page, S., Cusack, P., Frith, D., and Trott, D. (2020). Antimicrobial use and stewardship practices on Australian beef feedlots. *Australian Veterinary Journal* **98**, 37-47.

Bag, S., Ghosh, T. S., Banerjee, S., Mehta, O., Verma, J., Dayal, M., Desigamani, A., Kumar, P., Saha, B., Kedia, S., Ahuja, V., Ramamurthy, T., and Das, B. (2019). Molecular Insights into Antimicrobial Resistance Traits of Commensal Human Gut Microbiota. *Microb Ecol* **77**, 546-557.

Barlow, R., Mcmillan, K., Mellor, G., Duffy, L., Jordan, D., Abraham, R., O'dea, M., Sahibzada, S., and Abraham, S. (2022). Phenotypic and Genotypic Assessment of Antimicrobial Resistance in Escherichia coli from Australian Cattle Populations at Slaughter. *Journal of Food Protection* **85**, 563-570.

Bender, J. K., Cattoir, V., Hegstad, K., Sadowy, E., Coque, T. M., Westh, H., Hammerum, A. M., Schaffer, K., Burns, K., Murchan, S., Novais, C., Freitas, A. R., Peixe, L., Del Grosso, M., Pantosti, A., and Werner, G. (2018). Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resist Updat* **40**, 25-39.

Bergman, M., Nyberg, S. T., Huovinen, P., Paakkari, P., and Hakanen, A. J. (2009). Association between Antimicrobial Consumption and Resistance in Escherichia coli. *Antimicrobial Agents and Chemotherapy* **53**, 912-917.

CLSI (2020). Performance Standards for Antimicrobial Susceptibility Testing. *In* "Clinical and Laboratory Standards Institute", Vol. 30th ed.

Durso, L. M., and Cook, K. L. (2014). Impacts of antibiotic use in agriculture: what are the benefits and risks? *Curr Opin Microbiol* **19**, 37-44.

EFSA (2018). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA Journal* **16**, e05182.

Fauci, A. S., and Marston l, D. (2014). The perpetual challenge of antimicrobial resistance. *Jama* **311**, 1853-4.

Ho, P. L., Ng, K. Y., Lo, W. U., Law, P. Y., Lai, E. L., Wang, Y., and Chow, K. H. (2016). Plasmid-Mediated OqxAB Is an Important Mechanism for Nitrofurantoin Resistance in Escherichia coli. *Antimicrob Agents Chemother* **60**, 537-43.

Lancaster, H., Roberts, A. P., Bedi, R., Wilson, M., and Mullany, P. (2004). Characterization of Tn916S, a Tn916-like element containing the tetracycline resistance determinant tet(S). *J Bacteriol* **186**, 4395-8.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., and Monnet, D. L. (2012). Multidrug-resistant,

extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**, 268-81.

Melville, C. M., Brunel, R., Flint, H. J., and Scott, K. P. (2004). The Butyrivibrio fibrisolvens tet(W) gene is carried on the novel conjugative transposon TnB1230, which contains duplicated nitroreductase coding sequences. *J Bacteriol* **186**, 3656-9.

NARMS (2011). Retail Meat Annual Report. *FDA*.

Pokharel, S., Shrestha, P., and Adhikari, B. (2020). Antimicrobial use in food animals and human health: time to implement 'One Health' approach. *Antimicrobial Resistance & Infection Control* **9**, 181.

Schjørring, S., and Krogfelt, K. A. (2011). Assessment of bacterial antibiotic resistance transfer in the gut. *Int J Microbiol* **2011**, 312956.

Shakti, L., and Veeraraghavan, B. (2015). Advantage and limitations of nitrofurantoin in multi-drug resistant Indian scenario. *Indian J Med Microbiol* **33**, 477-81.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., Haesebrouck, F., and Butaye, P. (2010). Broad-spectrum β-lactamases among Enterobacteriaceae of animal origin: molecular aspects, mobility and impact on public health. *FEMS microbiology reviews* **34**, 295-316.

Tangcharoensathien, V., Chanvatik, S., and Sommanustweechai, A. (2018). Complex determinants of inappropriate use of antibiotics. *Bulletin of the World Health Organization* **96**, 141-144.

Vikram, A., Rovira, P., Agga, G. E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., Morley, P. S., Belk, K. E., and Schmidt, J. W. (2017). Impact of "Raised without Antibiotics" Beef Cattle Production Practices on Occurrences of Antimicrobial Resistance. *Appl Environ Microbiol* **83**.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science* **279**, 996-7.

7. Appendix – Supplementary Tables

Table S1. Antimicrobial resistance prevalence (% of isolates) in *Escherichia coli* isolated from rectal swabs collected at the abattoir (n=106)

	Antimicrobial used (Number of cattle)					
Antimicrobials	**CTC200 (14)**	**CTC200_Bovatil (42)**	**CTC200_Draxxin (27)**	**Draxxin_Bivatop200 (2)**	**CTC200_Bovatil_Draxxin (10)**	**CTC200_Draxxin_Bivatop200 (11)**
Ampicillin	0	16.67	7.41	50	0	9.09
Augmentin	0	0	0	0	0	0
Azithromycin	0	0	0	0	0	0
Cefoxitin	0	0	0	0	0	0
Ceftiofur	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	0	0
Chloramphenicol	0	2.38	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0
Gentamycin	0	0	0	0	0	0
Nalidixic acid	0	0	0	0	0	0
Streptomycin	14.29	7.14	3.7	0	0	9.09
Sulfisoxazole	14.29	9.52	7.41	0	10	9.09
Tetracycline	85.71	76.19	51.85	50	60	54.55
Trim/Sulfa	0	4.76	0	0	0	0

Table S2. The antimicrobial susceptibility testing results for *Enterococcus* spp. isolated from treated cattle before their first treatment

Antimicrobial class	Antimicrobial agent	Species (n)	Resistance (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)																
					0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Aminoglycosides	Gentamycin	EFAE (45)	0.0	-														100.0				
		EHIR (12)	0.0	-														100.0				
		Others (18)	0.0	-														100.0				
	Kanamycin	EFAE (45)	2.2	0.76-3.68														93.3	2.2	2.2		2.2
		EHIR (12)	0.0	-														100.0				
		Others (18)	0.0	-														100.0				
	Streptomycin	EFAE (45)	2.2	0.76-3.68																97.8		2.2
		EHIR (12)	0.0	-																100.0		
		Others (18)	0.0	-																100.0		
Beta lactam	Penicillin	EFAE (45)	0.0	-					8.9		20.0	40.0	31.1									
		EHIR (12)	0.0	-					50.0	25.0	16.7	8.3										
		Others (18)	0.0	-					5.6	44.4	38.9	11.1										
Fluoroquinolones	Ciprofloxacin	EFAE (45)	35.6	34.10-37.00					2.2	8.9	17.8	35.6	33.3	2.2								
		EHIR (12)	0.0	-					8.3	91.7												
		Others (18)	0.0	-					5.6	72.2	22.2											
Glycopeptides	Vancomycin	EFAE (45)	0.0	-					4.4	80.0	4.4	11.1										
		EHIR (12)	0.0	-						75.0	25.0											
		Others (18)	0.0	-					5.6	94.4												
Glycylcyclines	Tigecycline	EFAE (45)	0.0	-				51.1	48.9													
		EHIR (12)	0.0	-				58.3	41.7													
		Others (18)	5.6	3.25-7.87				50.0	44.4	5.6												
Lincosamide	Lincomycin	EFAE (45)	53.3	51.90-54.80							40.0	4.4	2.2	2.2	51.1							
		EHIR (12)	100.0	97.20-100.00											100.0							

		Others (18)	88.9	86.60-91.20			5.6	5.6		11.1	77.8					
Lipopeptides	Daptomycin	EFAE (45)	15.6	14.10-17.00	2.2	2.2		13.3	66.7	15.6						
		EHIR (12)	16.7	13.80-19.50			8.3	25.0	50.0	16.7						
		Others (18)	0.0	-	5.6	11.1	11.1	72.2								
Macrolides	Erythromycin	EFAE (45)	26.7	25.20-28.10	6.7	8.9	20.0	37.8			26.7					
		EHIR (12)	16.7	13.80-19.50	83.3						16.7					
		Others (18)	5.6	3.25-7.87	61.1	16.7	16.7				5.6					
	Tylosine tartrate	EFAE (45)	24.4	23.00-25.90		2.2	4.4	37.8	26.7	4.4			24.4			
		EHIR (12)	16.7	13.80-19.50				75.0	8.3				16.7			
		Others (18)	5.6	3.25-7.87			44.4	27.8	22.2				5.6			
Nitrofurantoins	Nitrofurantoin	EFAE (45)	4.4	2.98-5.90				2.2			2.2	8.9	82.2	4.4		
		EHIR (12)	0.0	-								50.0	50.0			
		Others (18)	5.6	3.25-7.87					5.6		16.7	33.3	38.9	5.6		
Oxazolidinones	Linezolid	EFAE (45)	0.0	-		4.4	55.6	40.0								
		EHIR (12)	0.0	-			58.3	41.7								
		Others (18)	0.0	-		5.6	61.1	33.3								
Phenicols	Chloramphenicol	EFAE (45)	6.7	5.21-8.13				2.2	84.4	2.2	4.4	6.7				
		EHIR (12)	0.0	-					100.0							
		Others (18)	0.0	-					94.4		5.6					
Streptogramins	Quinupristin /dalfopristin	EFAE (45)	17.8	16.30-19.20		53.3	4.4	24.4	4.4	2.2	2.2	8.9				
		EHIR (12)	0.0	-			25.0	75.0								
		Others (18)	5.6	3.25-7.87			27.8	66.7			5.6					
Tetracycline	Tetracycline	EFAE (45)	71.1	69.70-72.60			28.9					13.3	57.8			
		EHIR (12)	75.0	72.20-77.80			25.0					8.3	66.7			
		Others (18)	55.6	53.30-57.90			44.4					11.1	44.4			

Table S3. Antimicrobial susceptibility test results in Enterococcus spp. isolated from rectal swabs from apparently healthy cattle at the abattoir

Antimicrobial class	Antimicrobial agent	Species (n)	Resistance (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)																
					0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Aminoglycosides	Gentamycin	EFAE (14)	0.0	-														100.0				
		EHIR (36)	0.0	-														100.0				
		Others (5)	0.0	-														100.0				
	Kanamycin	EFAE (14)	0.0	-														100.0				
		EHIR (36)	0.0	-														100.0				
		Others (5)	0.0	-														100.0				
	Streptomycin	EFAE (14)	0.0	-																100.0		
		EHIR (36)	0.0	-																100.0		
		Others (5)	0.0	-																100.0		
Beta lactam	Penicillin	EFAE (14)	0.0	-								21.4	78.6									
		EHIR (36)	0.0	-					38.9	33.3	16.7	8.3	2.8									
		Others (5)	0.0	-					20.0	60.0	20.0											
Fluoroquinolones	Ciprofloxacin	EFAE (14)	14.3	11.70-16.90							21.4	64.3	14.3									
		EHIR (36)	0.0	-					30.6	66.7	2.8											
		Others (5)	0.0	-					20.0	40.0	40.0											
Glycopeptides	Vancomycin	EFAE (14)	0.0	-						100.0												
		EHIR (36)	0.0	-						88.9	11.1											
		Others (5)	0.0	-					20.0	80.0												
Glycylcyclines	Tigecycline	EFAE (14)	14.3	11.70-16.90			7.1	42.9	35.7	14.3												
		EHIR (36)	2.8	1.15-4.41		2.8	11.1	66.7	16.7	2.8												
		Others (5)	0.0	-				80.0	20.0													
Lincosamide	Lincomycin	EFAE (14)	78.6	75.90-81.20							21.4			7.1	71.4							
		EHIR (36)	91.7	90.00-93.30							2.8		5.6	2.8	88.9							
		Others (5)	100.0	95.60-100.00											100.0							

Lipopeptides	Daptomycin	EFAE (14)	7.1	4.52-9.76				7.1	85.7	7.1				
		EHIR (36)	27.8	26.20-29.40			8.3	13.9	50.0	27.8				
		Others (5)	0.0	-		20.0	60.0	20.0						
Macrolides	Erythromycin	EFAE (14)	0.0	-	50.0	7.1	7.1	35.7						
		EHIR (36)	19.4	17.80-21.10	72.2			2.8	5.6		19.4			
		Others (5)	0.0	-	60.0	40.0								
	Tylosine tartrate	EFAE (14)	0.0	-				28.6	21.4	50.0				
		EHIR (36)	27.8	26.20-29.40			2.8	69.4					27.8	
		Others (5)	0.0	-				100.0						
Nitrofurantoins	Nitrofurantoin	EFAE (14)	50.0	47.40-52.60									50.0	50.0
		EHIR (36)	2.8	1.15-4.41								58.3	38.9	2.8
		Others (5)	0.0	-							20.0	20.0	60.0	
Oxazolidinones	Linezolid	EFAE (14)	0.0	-			7.1	92.9						
		EHIR (36)	0.0	-			61.1	38.9						
		Others (5)	0.0	-			20.0	80.0						
Phenicols	Chloramphenicol	EFAE (14)	0.0	-					92.9	7.1				
		EHIR (36)	0.0	-					97.2	2.8				
		Others (5)	0.0	-					100.0					
Streptogramins	Quinupristin /dalfopristin	EFAE (14)	0.0	-		28.6		71.4						
		EHIR (36)	0.0	-		2.8	19.4	77.8						
		Others (5)	0.0	-				100.0						
Tetracycline	Tetracycline	EFAE (14)	7.1	4.52-9.76			92.9						7.1	
		EHIR (36)	50.0	48.40-51.60			47.2	2.8					50.0	
		Others (5)	0.0	-			100.0							

Table S4. Antimicrobial susceptibility tests in enterococci isolated from rectal samples from treated cattle collected at the abattoir

Antimicrobial class	Antimicrobial agent	Species (n)	Resistance (%)	CI (95 %)	MIC value (µg/mL) and Isolates (%)																
					0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
		EFAE (11)	0.0	-														100.0				
	Gentamycin	EHIR (37)	0.0	-														100.0				
		Others (4)	0.0	-														100.0				
		EFAE (11)	0.0	-														90.9	9.1			
Aminoglycosides	Kanamycin	EHIR (37)	0.0	-														100.0				
		Others (4)	0.0	-														100.0				
		EFAE (11)	0.0	-																100.0		
	Streptomycin	EHIR (37)	0.0	-																100.0		
		Others (4)	0.0	-																100.0		
		EFAE (11)	0.0	-								18.2	81.8									
Beta lactam	Penicillin	EHIR (37)	0.0	-					37.8	37.8	16.2	8.1										
		Others (4)	0.0	-					50.0	25.0	25.0											
		EFAE (11)	9.1	6.14-12.00							63.6	27.3	9.1									
Fluoroquinolones	Ciprofloxacin	EHIR (37)	0.0	-					27.0	70.3	2.7											
		Others (4)	0.0	-						75.0	25.0											
		EFAE (11)	0.0	-						100.0												
Glycopeptides	Vancomycin	EHIR (37)	0.0	-						78.4	21.6											
		Others (4)	0.0	-						100.0												
		EFAE (11)	9.1	6.14-12.00			9.1	63.6	18.2	9.1												
Glycylcyclines	Tigecycline	EHIR (37)	10.8	9.20-12.40			5.4	48.7	35.1	10.8												
		Others (4)	0.0	-				75.0	25.0													
		EFAE (11)	63.6	60.70-66.60							27.3	9.1			63.6							
Lincosamide	Lincomycin	EHIR (37)	86.5	84.90-88.10							10.8		2.7	2.7	83.8							
		Others (4)	75.0	70.10-79.90									25.0		75.0							

		EFAE (11)	18.2	15.20-21.10				18.2	63.6	18.2						
Lipopeptides	Daptomycin	EHIR (37)	29.7	28.10-31.30			8.1	18.9	43.2	27.0	2.7					
		Others (4)	0.0	-	25.0	25.0		50.0								
		EFAE (11)	9.1	6.14-12.00	54.6		9.1	27.3			9.1					
	Erythromycin	EHIR (37)	29.7	28.10-31.30	64.9			2.7	2.7		29.7					
		Others (4)	0.0	-	75.0		25.0									
Macrolides		EFAE (11)	9.1	6.14-12.00				27.3	9.1	54.6			9.1			
	Tylosine tartrate	EHIR (37)	35.1	33.50-36.80			2.7	62.2					35.1			
		Others (4)	0.0	-			50.0	25.0		25.0						
		EFAE (11)	9.1	6.14-12.00									90.9	9.1		
Nitrofurantoins	Nitrofurantoin	EHIR (37)	2.7	1.09-4.31							2.7	64.9	29.7	2.7		
		Others (4)	25.0	20.10-29.90					25.0				50.0	25.0		
		EFAE (11)	0.0	-				100.0								
Oxazolidinones	Linezolid	EHIR (37)	0.0	-			48.7	51.4								
		Others (4)	0.0	-			25.0	75.0								
		EFAE (11)	0.0	-					63.6	36.4						
Phenicols	Chloramphenicol	EHIR (37)	0.0	-				2.7	83.8	13.5						
		Others (4)	0.0	-					100.0							
		EFAE (11)	27.3	24.30-30.20		27.3		45.5	18.2	9.1						
Streptogramins	Quinupristin /dalfopristin	EHIR (37)	0.0	-		10.8	29.7	59.5								
		Others (4)	0.0	-			50.0	50.0								
	Tetracycline	EFAE (11)	9.1	6.14-12.00			90.9					9.1				
Tetracycline		EHIR (37)	40.5	38.90-42.10			59.5						40.5			
		Others (4)	25.0	20.10-29.90			75.0					25.0				

Table S5. Prevalence of antimicrobial resistance in *Enterococcus hirae* isolated from rectal swabs collected at the abattoir (n=73)

	Antimicrobial used (Number of cattle)				
Antimicrobials	**CTC200 (3)**	**CTC200+Bovatil (33)**	**CTC200+Draxxin (20)**	**CTC200+Bovatil_Draxxin (7)**	**CTC200+Draxxin+Bivatop200 (10)**
Chloramphenicol	0	0	0	0	0
Ciprofloxacin	0	0	0	0	0
Daptomycin	33.33	27.27	25	42.86	30
Erythromycin	0	21.21	15	42.86	50
Gentamicin	0	0	0	0	0
Kanamycin	0	0	0	0	0
Lincomycin	100	90.91	90	71.43	90
Linezolid	0	0	0	0	0
Nitrofurantoin	0	3.03	5	0	0
Penicillin	0	0	0	0	0
Quinupristin/dalfopristin	0	0	0	0	0
Streptomycin	0	0	0	0	0
Tetracycline	33.33	51.52	35	42.86	50
Tigecycline	0	3.03	15	0	10
Tylosin tartrate	0	30.3	20	57.14	50
Vancomycin	0	0	0	0	0

Table S6. Prevalence of antimicrobial resistance in *Enterococcus faecium* isolated from rectal swabs collected at the abattoir (n=25).

	Antimicrobial used (Number of cattle)				
Antimicrobials	**CTC200 (8)**	**CTC200+Bovatil (6)**	**CTC200+Draxxin (6)**	**CTC200+Bovatil+Draxxin (2)**	**CTC200+Draxxin+Bivatop200 (3)**
Chloramphenicol	0	0	0	0	0
Ciprofloxacin	12.5	0	0	50	0
Daptomycin	0	16.67	33.33	0	0
Erythromycin	0	0	16.67	0	0
Gentamicin	0	0	0	0	0
Kanamycin	0	0	0	0	0
Lincomycin	100	50	66.67	0	100
Linezolid	0	0	0	0	0
Nitrofurantoin	50	50	16.67	0	0
Penicillin	0	0	0	0	0
Quinupristin/dafloprístin	0	0	33.33	0	33.33
Streptomycin	0	0	0	0	0
Tetracycline	0	16.67	0	50	0
Tigecycline	25	0	0	0	33.33
Tylosin tartrate	0	0	16.67	0	0
Vancomycin	0	0	0	0	0

Table S7. Distribution of sampled cattle per feedlot pen

Pen	Treated	Non-treated	Total
1	3	4	7
2	7	6	13
3	5	7	12
4	5	7	12
5	7	6	13
6	5	6	11
7	6	7	13
8	4	5	9
9	8	7	15
10	7	5	12
11	6	7	13
Total	**63**	**67**	**130**

8. Appendix – Literature review

Literature review on the effect of ionophores on antimicrobial resistance of the microbiome, *Escherichia coli, Salmonella* and *Enterococcus* in beef cattle and wider food animal production

Abstract

Monensin is a member of the polyether ionophore antimicrobials (hereafter referred to as ionophores) approved for use as a modifier of rumen microbiota in cattle in Australia and elsewhere. The aim of this review is to summarise literature with emphasis on the ionophore mechanism of action and critical analysis of its role (perceived or otherwise) in the development of antimicrobial resistance of risk to human health. The literature related to the use of the ionophore monensin (and others), and resistance profiles of indicator bacteria, particularly in manure of beef cattle was reviewed. The primary mode of ionophores in modifying rumen microbiota is by competitive selection for a higher proportion of Gram-negative bacteria (via growth inhibition of selected Gram-positive bacteria) as well as mild inhibition of protozoan populations. The modified rumen microbiota decreases energy loss through reduced methane production and partially abates the heat stress effects on cattle, particularly when combined with tannins. Ionophores, including monensin, increase production of propionate and decrease methane production and emission by approximately 25% through reduced availability of hydrogen and formate; hence, increasing energy utilisation in the rumen; although effects on feed conversion ratio vary from insignificant to moderate. Furthermore, ionophores depress microbial utilisation of protein, increasing both rumen bypass protein and the availability of gut protein to cattle. Dietary ionophores decrease the risk of bloat and rumen acidosis and the mobilisation of body adipose tissues in early lactation with resulting decreased prevalence of ketosis and displaced abomasa; hence, they provide direct health benefits for cattle. Additionally, the use of monensin has been associated with decreased faecal shedding of *Escherichia coli* O157:H7 that indicates decreased risk of human food safety issues. However, monensin may be detected in fresh

manure and may be regarded in some quarters as a potential environmental pollutant. The current understanding is that monensin in cattle manure has a positive effect on the carbon footprint, as it decreases the production of methane and nitrogen gasses and water contamination with nitrogen from manure and runoff surface waters. The role of ionophores in human clinical medicine does not present many concerns with its ongoing use in agriculture. There are indications that ionophores may be used as anticancer therapy. Some ionophores, from the group zinc ionophores are currently being investigated as antimicrobials or even immune stimulators against COVID-19 viral infections. Resistance to ionophores has been reported among Gram-positive bacteria of clinical significance including coagulase-negative staphylococci isolated from cattle and also in *Enterococcus faecium* and *E. faecalis.* Despite the relatively long history of use for over five decades, the level of reported ionophore resistance is still miniscule. In conclusion, the use of monensin in beef feedlot cattle is unlikely to have a significant effect on the development and dissemination of antimicrobial resistance determinants of clinical significance from cattle to humans.

General Background

Antimicrobial resistance is a severe and mounting OneHealth concern throughout the globe. At present, the successful management of many diseases affecting human health depends greatly on the use of antimicrobials. Unfortunately, antimicrobial resistance has rendered some of these therapeutic options fruitless, for example the successful treatment of sepsis caused by pan-resistant Gram-negative pathogens. This coupled with the extremely low pace of development of new antimicrobial agents, makes the expediency of antimicrobial therapy uncertain. The challenge is to extend the useful life of existing antimicrobial classes whilst new drugs are developed. This can be achieved by antimicrobial stewardship in human and veterinary medicine coupled with arresting non-clinical pathways of development and spread of antimicrobial resistance. However, the non-clinical pathways of antimicrobial resistance are currently not suitably addressed by the World Health Organisation (WHO, 2015).

In spite of the conflicted opinions regarding concrete scientific evidence that show antimicrobial resistance transfer (to humans) of bacteria associated either with animals fed non-treatment antimicrobials (NTAs), or their food products, some researchers have noted the possibility of antimicrobial resistance determinants arising in food animals and transferring to bacterial pathogens of humans indirectly (Witte Wo et al., 2000). For example, the spread of resistance, as it applies to enterococci in the human gastrointestinal via consumption of meat products and/or vegetable crops grown via the application of animal manure, relates to the dissemination of the *vanA* gene cluster integrated into different conjugative plasmids among a variety of different *Enterococcus faecium*

strains (Biavasco et al., 2007). Streptogramin resistance associated with *vat* genes has also been found in *E. faecium* of animal and human clinical origin (Jung et al., 2010), and because virginiamycin has historically been used as growth promoter in animals whereas streptogramins have been used infrequently in human medicine, some researchers have suggested an animal origin of resistance (Gouliouris et al., 2018). However, whilst the human gastrointestinal tract may be colonised by animal-origin *Enterococcus*, they are genetically distinct and carry different resistance genes when compared with human clinical isolates (Gouliouris et al., 2018).

In the last few decades, it has become obvious that the greater environment may serve as a reservoir for antimicrobial resistance determinants (Storteboom et al., 2007; Tripathi and Tripathi, 2017; Tyrrell et al., 2019). Use of antimicrobials for human and animal health are closely linked, either directly via contact or indirectly through the environment. Hence, in this review, we will address all One Health aspects concerning the development and spread of antimicrobial resistance related to ionophore use in cattle. The use of ionophores as antimicrobial chemotherapeutic, antineoplastic and immunomodulatory agents in humans is beyond the scope of this review and it is not further discussed.

There have been many conflicted opinions regarding bona fide scientific evidence on antimicrobial resistance transfer in bacteria associated either with animals fed NTAs, or their food products to humans. Angulo et al. (2004) highlighted concerns regarding resistance determinant transmission from food animals to humans, especially those encoding resistance to antimicrobial agents that were used in food animals for growth promotion, as they may increase the likelihood that human bacterial pathogens that have food animal reservoirs, will develop cross-resistance to drugs approved for use in human medicine (Angulo et al., 2004). Marshall and Levy (2011) reported the need for eliminating NTA use in food animals in order to reduce the growing environmental load of resistance genes and its possible spread to other animals and humans-directly by contact and indirectly via the food chain, water, air, and manured and/or sludge-fertilised soils (Marshall and Levy, 2011).

Ionophores provide economic and environmental benefits for the beef industry as they reduce methane production, allow the rumen to utilize feed energy and protein more efficiently to reduce the risk of bloat. Ionophores are not listed as important in human medicine, and there is currently no evidence that their use in livestock increases resistance to other antimicrobials that are important in cattle or human medicine. Furthermore, ionophore resistance does not appear to pass from one generation of bacteria to another. This probably explains why ionophores are still effective after being heavily used in beef production for the last 50 years.

Ionophores act by disrupting the ion concentration gradient (Ca^{2+}, K^{+}, H^{+}, Na^{+}) across lipid bilayer membrane of Gram-positive bacteria. This disruption prevents the microorganisms from maintaining normal metabolism and causes them to expend additional energy (Hersom & Thrift, 2018). This negative function of ionophores selectively affects the microorganisms that decrease efficient digestive physiology within the rumen, eventually leading to the modulation of certain protozoa and bacteria in the rumen with associated reduction in metabolic end products with methane in particular (Guan et al. 2006). This unique mechanism of action shifts the rumen microbiome to allow beneficial bacteria to become more dominant and efficient through increased propionic acid and decreased acetic acid and lactic acid production from suppression of specific genera (Hersom & Thrift, 2018) such as members of the *Streptococcus bovis / Streptococcus equinus* complex ((Chow and Russell, 1990)). Ionophores can be fed to cattle in many different ways; either included in dry or liquid manufactured supplements, or in loose mineral mixtures. For example, supplementation with 155 mg/day of monensin resulted in an improved average daily gain of 0.8 kg/day or a 13.5% increase compared to non-supplemented control cattle (Kunkle et al. 2000). Offering supplements containing monensin at 200 or 400 mg/day on alternate days can increase growing calf gain by 0. 8 kg/day, respectively (Muller et al., 1986). Additionally, cattle grazing Bermuda grass and supplemented with 200 mg/day of monensin in the summer have been reported to increase daily gain by 0.1 – 0.2 kg/day or a 24%–44% increase over cattle consuming supplement without monensin (Oliver, 1975).

Antimicrobial use in animals and its impact to human health

Production of ionophores is reliant on industrial fermentation of particular genera from the *Streptomycetacae* family, mainly *Actinomadura, Dactylosporangium* and *Streptomyces* spp. (Kevin li et al., 2009). Monensin has a long history of use in food animals, being the first marketed ionophore of veterinary importance. With respect to ionophores, researchers (Callaway et al., 2003; Yoshida et al., 2010) have concluded that their use in beef cattle is unlikely to cause significant effects on human clinical medicine. In 2003, Russell and Houlihan concluded no significant impacts on human health of ionophore use in animal feed on the transfer of antimicrobial resistance genes from animals to humans. Additionally, ionophores may prevent transfer of plasmid-mediated antimicrobial resistance determinants to sensitive bacterial strains (Mathers et al., 2004), a characteristic that may actually prolong the lifespan of usage of antimicrobials before significant resistance develops. Uptake of antimicrobials (e.g., chlortetracycline, monensin, sulfamethazine, tylosin, and virginiamycin) by 11 vegetable crops grown in different soils and fertilized with raw versus composted animal manures or inorganic fertilizer and concluded minimal human health risks have been assessed (Kang et al., 2013; Russell and Houlihan, 2003).

Polyether ionophores and monensin

Monensin is a member of the polyether ionophore antimicrobials which are approved for use as rumen microbiota modifiers in cattle in Australia and elsewhere (Azzaz et al., 2015; Kevin Ii et al., 2009). Ionophores are the most common antimicrobials administered to beef cattle in many developed countries (Noyes et al., 2016a; Yoshida et al., 2010). Currently, ionophores are not listed as critically important to human clinical medicine (Butaye et al., 2003; Hudson et al., 2017; Noyes et al., 2016a).

All natural ionophores are produced by bacteria of *Streptomycetaceae* family (Kevin Ii et al., 2009; Tedeschi et al., 2003; Yoshida et al., 2010). Ionophores as carboxyl polyethers, and have pronounced activity against bacteria, fungi, protozoa, and viruses (Azzaz et al., 2015; Kevin Ii et al., 2009). They also express some anti-inflammatory, antineoplastic, cardio-vascular- or CNS-modulatory, herbicidal, immunoregulatory, and insecticidal activity (Kevin Ii et al., 2009). Interest in ionophore use in human medicine have resurfaced recently due to their potential activity against multidrug-resistant (MDR) pathogens and advanced cancers (Kaushik et al., 2018; Kevin Ii et al., 2009). The potential use of ionophores in antimicrobial chemotherapy for treatment of multidrug-resistant infections (e.g., against methicillin-resistant *Staphylococcus aureus* – MRSA and vancomycin-resistant enterococci - VRE) has gained recent interest but has to be balanced by their systemic toxicity (Kevin Ii et al., 2009). Furthermore, research on antimicrobial and immune-modulating properties of ionophores is ongoing, including proposed activity against viruses as applied to COVID-19 infections (Bohlmann et al., 2018; Cingolani, 2021; De Oliveira et al., 2020; Harbison-Price et al., 2020; Sigle et al., 2006).

Use of ionophores in cattle

Ionophores in cattle are used to improve feed conversion efficiency resulting in improved production (meat and/or milk) and reduced morbidity and mortality (Azzaz et al., 2015). These benefits are achieved through rumen microbiota modifications. The primary mode of modification of rumen microbiota by ionophores is by competitive selection for higher proportion of Gram-negative bacteria in the rumen contents by inhibiting the growth of selected Gram-positive bacteria (e.g. members of the *Streptococcus bovis/equinum* complex) and a mild inhibition of protozoa. The modified rumen microbiota decreases energy loss via methane production and partially abates the heat stress effects on cattle, particularly when combined with tannins. Ionophores, including monensin, increase production of propionate and decrease methane production and emission by up to 25% (Azzaz et al., 2015; Hao et al., 2014; Odongo et al., 2007; Place et al., 2011; Ranga Niroshan Appuhamy et al., 2013; Tedeschi et al., 2003) through suppression of the availability of hydrogen and formate (Azzaz et al.,

2015); hence, increasing energy utilisation in the rumen (Azzaz et al., 2015; Place et al., 2011). The volatile fatty acid changes are predominantly an increase in propionate and decrease in lactate (Dennis et al., 1981; Hao et al., 2014). Ionophore supplementation results in decreased production of methane with minimal to no effect on the rumen degradation of fibrous material (Azzaz et al., 2015; Matthews et al., 2019; Place et al., 2011; Tedeschi et al., 2003). Furthermore, ionophores depress microbial utilisation of protein and reduction of ammonia resulting in an increase in the level of rumen bypass protein and gut protein reaching the abomasum and intestines (Azzaz et al., 2015; Lana et al., 1997; Tedeschi et al., 2003). The change in rumen fermentation and fermentation products is mediated by a partial shift in the rumen microbiota in favour of Gram-negative bacteria and decrease in ciliated protozoa (Hao et al., 2014; Tedeschi et al., 2003). These changes may affect the feed conversion ratio though benefits are variable and ranged from insignificant to moderate increases (Azzaz et al., 2015; Benchaar et al., 2006; Lana et al., 1997; Tedeschi et al., 2003).

Dietary ionophores provide a number of health benefits for all ruminants, including cattle. Through control of rumen fermentation, they decrease the risk of bloat and rumen acidosis (Azzaz et al., 2015; Place et al., 2011; Tedeschi et al., 2003). Dietary ionophores also decrease the mobilisation of body adipose tissues in early lactation resulting in lower prevalence of ketosis and displaced abomasa (Tedeschi et al., 2003). In cattle used in reproduction, ionophores, particularly monensin, have shown decreases in the days to conception, thereby decreasing the risk of involuntary culling, particularly in seasonal calving populations (Tedeschi et al., 2003)

Finally, dietary ionophores also provide a number of environmental benefits. The decreased methane production and greenhouse gas emissions can certainly be considered as important global OneHealth strategy for mitigating climate change (Tedeschi et al., 2003). The decreased nitrogen requirement and losses through faces and urine are also a significant environmental benefit (Tedeschi et al., 2003). Reduced nitrogen emissions have positive effects on human health and marine ecosystems. The increased feed conversion ratio means that less dietary resources need to be used (Tedeschi et al., 2003) and the amount of manure produced from cattle is decreased (Tedeschi et al., 2003). The use of ionophores, particularly monensin, allows feeding of by-products with a higher fat content that would otherwise suppress rumen fermentation, decreasing the waste from various industries (e.g., cotton, ethanol) (Tedeschi et al., 2003). Additionally, the use of monensin has been associated with decreased faecal shedding of *Escherichia coli* O157:H7 that indicates decreased human food safety risks.

Mechanism of action of ionophores

The name ionophores derives from their capacity to bind to cations and facilitation transport across the membranes of various cells (Kevin Ii et al., 2009). Therefore, they can be considered as toxic to both eukaryotic and prokaryotic cells (Arikan et al., 2016; Butaye et al., 2003).

Mechanism of action of polyether ionophores is not completely understood. It is believed their unique interaction with metal cations (Ca^{++}, K^{+}, Mg^{++}, and Na^{+}) coupled with their lipophilicity underpins their antimicrobial activity (Azzaz et al., 2015; Butaye et al., 2003; Kevin Ii et al., 2009; Pressman, 1976). Cationic affinity results in a paracyclic metallo-lipid complex with head-to-tail bonding to hydrogen, leading to altered intracellular cation and electron balances (Hoogerheide and Popov, 1979; Kevin Ii et al., 2009; Pressman, 1976). The presence of an outer membrane, which is believed to be impermeable to the hydrophobic compounds, imparts intrinsic resistance to ionophores in most Gram-negative bacteria, with some exceptions (Butaye et al., 2003; Kevin Ii et al., 2009).

Each ionophore has a unique cationic affinity and lipophilicity that affects its antimicrobial activity. As lasalocid, monensin and narasin are used in cattle, their ion preferences will be briefly described. Lasalocid is effective in transporting divalent ions (Ca^{++} and Mg^{++}) but also K^{+} (Butaye et al., 2003). Monensin has a high affinity to Na^{+} and disrupts osmotic pressure and energy utilisation in the affected cells (Butaye et al., 2003; Watanabe et al., 2008). Narasin is an efficient K^{+} carrier (Butaye et al., 2003).

Known spectrum of activities of polyether ionophores

Ionophores have a good Gram-positive and limited Gram-negative spectrum of activity, and particular activity is reported against anaerobes (Kevin Ii et al., 2009; Nagaraja and Taylor, 1987; Newbold et al., 1988; Watanabe et al., 1981) with some specific differences. For example laidlomycin has been reported to be inactive against *S. aureus* whereas the other ionophores are generally active again this species (Kevin Ii et al., 2009). Moreover, some ionophores have broad spectrum, including some Gram-negative bacteria, such as mutalomycin, noboritomycin and septamycin (Kevin Ii et al., 2009). Depression of rumen methane production (Guan et al., 2006; Wildenauer et al., 1984) is dose-dependent (McGarvey et al., 2018). However, rumen microbiota may adapt to ionophores and methane production may not be as depressed as calculated in the early phases of exposure. Ionophores may also have a direct effect on the metabolism of cattle (Armstrong and Spears, 1988). Effects on manure microbiota with potential risk to human health does not appear to be evident by both decreased prevalence of *E. coli* O157:H7 (by an unknown mechanism) and no significant difference in the antimicrobial resistance profiles of indicator bacteria (Edrington et al., 2006) compared to non-exposed populations of cattle.

Antimicrobials in the environment

Antimicrobials may enter the ecosystem from various sources: 1. Aquaculture farming; 2. Crop production; 3. Disposal of expired medications; 4. Feedlot-raised animal production (e.g., cattle and sheep); 5. Intensive-animal production (e.g. pigs and poultry); 6. Pasture-raised animal production; 7. Pollution from the pharmaceutical industry; 8. Sewage, particularly from hospitals; and 9. Urban biosolids (Hudson et al., 2017; Quaik et al., 2020; Sanderson et al., 2016; Sassman and Lee, 2007; Storteboom et al., 2007; Tasho and Cho, 2016; Tyrrell et al., 2019; Watanabe et al., 2008). Therefore, antimicrobial resistance determinants may originate from agricultural, animal or human use (Acar and Moulin, 2006; Adator et al., 2020; Kemper, 2008), with reports of a significant clustering by origin of determinants (Adator et al., 2020; Agga et al., 2015). In animal production systems in developed countries with regulated antimicrobial use, the highest risk of antimicrobials entering the ecosystem exists with concentrated animal feeding operations (**CAFO**), including beef feedlots. One of the most important links between CAFOs and the ecosystem is the manure. The effect of the antimicrobial on the ecosystem depends on the species of animal the antimicrobial has been administered to, route, frequency and duration of administration, degradation rate of the antimicrobial in the environment, likelihood of the antimicrobial resistance determinants being transferred to environmental bacteria, and the metabolic rate of the treated animal/s (Tasho and Cho, 2016; Tyrrell et al., 2019).

Manure from animal production as part of the integral waste management system, including beef feedlot manure, is an important organic amendment to agricultural soils although it may take years until organic composition is improved (Abbott et al., 2018; Cheng et al., 2019b; Kemper, 2008; Kuppusamy et al., 2018). The use of soil amendments is expected to increase in the future as the agricultural production intensifies and global warming takes its toll (Abbott et al., 2018; Du and Liu, 2012). As an important soil organic amendment, manure from the beef feedlot industry should be a wholesome product available to cropping industries. The use of antimicrobials in animals, which may include ionophores, has been suggested to affect the quality of this biological amendment (Granados-Chinchilla et al., 2020; Hudson et al., 2017; Ruuskanen et al., 2016; Watanabe et al., 2008), and may also be important for dispersion of antimicrobial resistance determinants in the ecosystem (Amarakoon et al., 2016; Cleary et al., 2016; Durso and Cook, 2014b; Ruuskanen et al., 2016; Tyrrell et al., 2019).

Although any use of antimicrobials may have an effect on the ecosystem, when used under 'antimicrobial stewardship guidelines' the effects in manure can potentially be minimised (Acar and Moulin, 2006; Du and Liu, 2012; Kuppusamy et al., 2018; Ma et al., 2019). Furthermore, manure treatment (e.g., anaerobic digestion, composting) may be used to decrease concentrations of

antimicrobials, and, in most cases, the prevalence of antimicrobial resistance determinants in this organic soil amendment (Arikan et al., 2009; Chen et al., 2018; Dolliver et al., 2008; Kim et al., 2011; Ma et al., 2019; Oliver et al., 2020; Pu et al., 2019; Qian et al., 2018; Ray et al., 2017). Indeed, the effect on the antimicrobial concentrations and antimicrobial resistance determinants varies between antimicrobials, type of manure and the type of manure treatment (Pu et al., 2019; Qian et al., 2018). For some antimicrobials, urine may also be of importance. However, for dietary ionophores, no urine excretion has been detected (Spielmeyer, 2018). Even when absorbed, ionophores undergoes hepatic metabolism and biliary excretion, ultimately resulting in monensin being contained to the alimentary system and completely eliminated with faeces (Donoho et al., 1978; Herberg et al., 1978).

Antimicrobials present in manure may enter the water ecosystem, either by direct or indirect contamination of waterways by manure or through wastewater run offs (Kemper, 2008; Kim et al., 2011; Netthisinghe et al., 2018; Watanabe et al., 2008). The effect of wastewater run offs may be important in farm ecosystems (Acar and Moulin, 2006; Du and Liu, 2012; Netthisinghe et al., 2018) but further work is definitely required (Chen et al., 2015; Cycoń et al., 2019; DeVries and Zhang, 2016). Although monensin could not be found in wastewater run offs from some beef feedlot farms (D'Alessio et al., 2019; Netthisinghe et al., 2018), it has been found in others (Sassman and Lee, 2007) as well as in wastewaters from other cattle industries (e.g. intensive dairy production) (Watanabe et al., 2010; Watanabe et al., 2008). Additionally, the spread of antimicrobial resistance determinants have been occasionally associated with the presence of subtherapeutic concentrations of some antimicrobials in wastewater run offs (also from dairy farms using monensin in the diet) (Amarakoon et al., 2014).

Finally, recent evidence has confirmed that pharmaceuticals, including antimicrobials may be transferred between ecosystems (e.g., beef feedlot and aquatic environments nearby) by air-borne particulate matter (e.g., dust) (Sandoz et al., 2018). This mode of contamination from beef feedlots should not be ignored.

Not all antimicrobial resistance determinants present within the environment are associated with feeding antimicrobials to livestock, including ionophores (Alexander et al., 2008; Rovira et al., 2019; Sanderson et al., 2016; Udikovic-Kolic et al., 2014). The presence of antimicrobial resistance determinants in the non-exposed populations has been lower than in exposed populations but by no means were non-exposed populations free of these determinants (Rovira et al., 2019; Sanderson et al., 2016). Some of the antimicrobial resistance determinants in soil are ancient and probably result from times when antimicrobials first appeared in soils naturally (Sanderson et al., 2016). However, the presence of these determinants cannot be entirely explained by natural mutations occurring in

soils. A clear relationship exist with the manufacture, and clinical and veterinary use of antimicrobials (Sanderson et al., 2016). Clearly, the level of antimicrobial resistance determinants is associated with the contamination of the ecosystem with antimicrobials (Sanderson et al., 2016; Tyrrell et al., 2019). The presence of antimicrobial resistance determinants in beef feedlot manure was in some cases hypothesised to be associated with environmental factors such as diet (Alexander et al., 2008; Noyes et al., 2016a; Noyes et al., 2016b; Rovira et al., 2019). Another hypothesised pathways is the re-use of the bedding in CAFOs (Tyrrell et al., 2019). Interestingly, amendment of soil by cattle manure resulted in increased antimicrobial resistance determinants to beta-lactams and tetracyclines in soils for several months, irrespective of the treatment exposure (Kyselková et al., 2013; Kyselková et al., 2015; Udikovic-Kolic et al., 2014) whereas the use of inorganic fertilisers did not change the presence of beta-lactamase resistance determinants (Udikovic-Kolic et al., 2014). This may pose a significant challenge to organic agriculture dependent on the use of organic manure as the only approved fertiliser for organic fields (Udikovic-Kolic et al., 2014).

Risks from antimicrobials in the ecosystem

Antimicrobials and antimicrobial resistance determinants from agricultural and aquatic environments may pose risks to humans. The level of these risks is yet to be confirmed. The risk of spread of antimicrobial resistance determinants comes from aerosol contamination, eating plants produced on the contaminated fields, drinking contaminated water or recreational use of contaminated waters (e.g., bathing, swimming) (Hudson et al., 2017; Pruden et al., 2013; Tyrrell et al., 2019). Plants may be contaminated by exposure to contaminated soils (e.g., amendment with contaminated manure) or irrigation with contaminated water (Hudson et al., 2017; Tyrrell et al., 2019). The risk of the presence of antimicrobials in plants or water lies in the possibility of allergic reactions, and in case of ionophores, the possibility of ionophore-associated toxicity. Toxicity may be particularly important for very susceptible animal species such equids, leporides and poultry (Butaye et al., 2003; Watanabe et al., 2008). The real risk of the presence of ionophores in edible plants is indeed negligible to low, as plants, dependent on the type, may bio-accumulate monensin in low to moderate concentrations (Kang et al., 2013; Tasho and Cho, 2016). However, antimicrobial resistance determinants could be found on some plants that were overhead irrigated with contaminated water (Shen et al., 2019). Ionophores present in wastewater used for irrigation of agricultural fields undergo a rapid decontamination in soils and effects of such contamination are minimal (Sassman and Lee, 2007). Moreover, the soil's adsorption and plant uptake of ionophores is minuscule (Watanabe et al., 2008). It must be noted that the presence of antimicrobials in the ecosystem and soils have negative effects on the soil

microbiota and iron utilisation, therefore decreasing the plant growth and crop performance (Toth et al., 2011).

Persistence of ionophores in manure, soil and other ecosystems

Persistence of antimicrobials in manure varies dependent on the types of both antimicrobial and manure, as well as manure treatment before land application (e.g., composting, lagoons or stock piling). Persistence of ionophores in stockpiled manure is limited and most of the residues degrade in the first week or two. Therefore, appropriate stock piling should prevent ionophore residues in manure used for fertilisation a month after being deposited (Oliver et al., 2020; Sassman and Lee, 2007; Yoshida et al., 2010). However, this does not apply equally to all ionophores and stock piling conditions. Half-life times for monensin in stock-pilled manure under anaerobic conditions are 30 – 70 days (Storteboom et al., 2007). Composting has shown better results in the degradation of ionophores. For monensin, insignificant degradation was detected with composting but the same procedure was efficacious for salinomycin with a short half-life of just over one day (Arikan et al., 2016; Donoho et al., 1978; Sassman and Lee, 2007; Storteboom et al., 2007; Youngquist et al., 2016). Contrary to this, composting resulted in a half-life of monensin of 30 days (Storteboom et al., 2007). Stock piling or composting may need to be extended as there were some indications that composting was slower in manure from cattle feed ionophore-enriched diets (Arikan et al., 2016; Cessna et al., 2011). Alternatively, manure can be enhanced by specific microbial inocula which facilitate degradation of antimicrobials and result in better compost for soil amendment (Li et al., 2020) or soil amendments (e.g., alum or biochar) which shorten monensin-related decontamination times (Netthisinghe et al., 2018). Amendments of lucerne hay and dry leaves, coupled with regular wetting and turning of the manure in the compost pile, halved the half-life for monensin from 30 to 15 days (Storteboom et al., 2007). Interestingly, pirlimycin-associated antimicrobial resistance determinants in lettuce grown on fields amended by fresh or composted manure or wastewater run offs from dairy cattle industry did not decrease after composting (Jacobs et al., 2019). Additionally, there is limited evidence that repeated amendment of the fields with manure may result in a build-up of the antimicrobial resistance to other antimicrobials (Udikovic-Kolic et al., 2014; Walczak and Xu, 2011; Wu et al., 2020).

Degradation of ionophores in soils was mainly attributed to biological activity, predominantly by various Gram-negative soil bacteria (Spielmeyer, 2018; Vertesy et al., 1987). Persistence of ionophores in soils is short, with a half-life of 3 – 28 days for ionophores (e.g., lasalocid, monensin and salinomycin), dependent on the presence of organic matter with more organic matter resulting in a shorter half-life (Carlson and Mabury, 2006; Dolliver et al., 2008; Gurmessa et al., 2020; Netthisinghe

et al., 2018; Sassman and Lee, 2007; Yoshida et al., 2010). The type of soil was also important for the degradation of ionophores, being about one week in clay-loam soils to three weeks in loam soils most likely due to different oxygen saturations (Yoshida et al., 2010). Finally, degradation varied with the soil moisture being slower in arid areas (Yoshida et al., 2010) and humidification being slower at lower temperatures (Storteboom et al., 2007). The variability in degradation times due to soil organic matter content, oxygen saturation and humidity can be explained by the differences in the soil microbiota required for ionophore degradation (Carlson and Mabury, 2006; Vertesy et al., 1987; Yoshida et al., 2010). Furthermore, ionophore concentration in soil may also influence the speed of degradation. For example, monensin concentrations in soils of previous beef backgrounding operations decreased rapidly whist in high concentrations, but later, in minute concentrations, degradation became much slower (Netthisinghe et al., 2018). Similarly, the reported half-life of monensin in wastewaters is 4 – 23 days (Watanabe et al., 2008).

The half-life of 3 - 28 days is slower than tetracycline but similar to sulphonamides and other medically important antimicrobials (Bailey et al., 2016; Dolliver et al., 2008; Oliver et al., 2020). However, in some studies the degradation of tetracyclines was much slower compared to ionophore-reported half-times, particularly in frozen soils (Amarakoon et al., 2016).

It should be noted that as all ionophores are naturally derived, the effect of monensin on the environmental microbiota is limited. For example, exposure to monensin results only in a transient and insignificant change to the biofilm-forming microbiota of freshwater (Winkworth and Lear, 2014). Additionally, fish reproduction is not affected by the presence of minute concentrations of various pharmaceuticals (Overturf et al., 2015). However, persistence of the pathogenic *E. coli* O157:H7 was prolonged from 0.8 to 5.1 days in samples of wastewater from dairy cattle lagoons that were fed monensin-containing diets (Ravva et al., 2013). Indeed, the presence of this pathogen may result in an increased risk to humans consuming plants originating from crops where these waters have been applied and this risk should not be ignored. However, this finding needs further investigation.

The degradation of an antimicrobial in the environment does not mean disappearance of the antimicrobial resistance determinants. They may persist for much longer in any particular environment (Oliver et al., 2020). Antimicrobial resistance may persist in part due to the low fitness cost related to many antimicrobial resistance determinants (Andersson and Hughes, 2010). This may be related also to the origin of the antimicrobial and its resistance mechanisms. As all ionophores are naturally derived from common soil microbes, their resistance determinants may not be foreign to soil bacteria and therefore, they may not disappear quickly or even at all. The effect on the structure and function of the soil microbiota may persist much longer than the presence of the antimicrobial

(Jechalke et al., 2014; Kemper, 2008; Kuppusamy et al., 2018; Netthisinghe et al., 2018). However, due to the complex relationship of various bacterial species in soils (Netthisinghe et al., 2018), estimation of the persistence of antimicrobial resistance determinants in soils is virtually impossible. Thus, the mere presence of an antimicrobial or antimicrobial resistance determinants in agricultural amendments does not translate into a direct risk to development or spread of antimicrobial resistance to humans. It is easy to blame animals and veterinary medicine but the evidence to substantiate these concepts is required. The evidence of the spread of animal-derived antimicrobial resistance determinants to humans through environmental contamination is yet to be substantiated although the risk should not be ignored (Ben et al., 2019; Cheng et al., 2019a; Heuer et al., 2011; Netthisinghe et al., 2018; Pan and Chu, 2017).

Antimicrobial resistance to ionophores

Antimicrobial resistance of some kind is reported for the majority of ionophores (Aarestrup et al., 1998; Butaye et al., 2003). However, there is lack of evidence that the resistance to ionophores can be easily transferred to humans, as the majority of resistance mechanisms are due to the presence of the outer membrane in Gram-negative bacteria which is intrinsic. Using non-standardised methodology, some resistance to monensin was detected in *Streptococcus hyicus, E. faecalis* and *E. faecium* originating from pig faeces (Aarestrup et al., 1998) and enterococci from pigs and poultry (Butaye et al., 2000). Narasin resistance is reported for poultry isolates of *Enterococcus faecium* in Sweden (Nilsson et al., 2016). Narasin, tetranasin and occasionally other ionophore resistances, mediated through ABC-transporter efflux pumps, is reported in a relatively large group of Gram-positive bacteria (Butaye et al., 2003; Linton et al., 1994; Naemi et al., 2020; Nilsson et al., 2016). The ABC-transporter efflux pump was associated with resistance to maduramicin, narasin and salinomycin but not monensin (Naemi et al., 2020). Salinomycin resistance is reported in *Clostridium difficile* but the mechanism is unknown (Hosseinzadeh et al., 2016).

Reversible adaptation in presence of monensin is reported for a few bacterial species of cattle origin, namely *E. faecium, E. faecalis* and *Clostridium perfringens* (Simjee et al., 2012). The adaptation is characterised by thickening of the cell membrane and/or changes in potassium exchange of bacterial strains of a reversible, non-genetically-encoded character. Moreover, resistance by an unexplained mechanism is confirmed for few bacterial species of cattle rumen origin, namely *Clostridium aminophilum* (Callaway et al., 1999; Rychlik and Russell, 2002), *Megasphaera elsdenii* (Callaway et al., 1999), *Prevotella ruminicola* (Callaway and Russell, 2000; Dawson and Boling, 1984; Newbold et al., 1993; Newbold et al., 1988), and *Selenomonas ruminantium* (Callaway et al., 1999). Monensin resistance, specifically, is also reported in a few bacterial species of porcine origin, namely

Staphylococcus hyicus and *Enterococcus* sp. It is important to mention that standardised testing methodology of the antimicrobial susceptibility may not be suitable for most ionophore-bacterial species combinations as the MIC values were affected by pH and various additives to the testing media (Butaye et al., 2000; Chow and Russell, 1990; Marounek and Rada, 1995).

For Gram-positive bacteria, the resistance has been reported against rumen-specific *Clostridium, Prevotella,* and *Streptococcus* spp. For example, resistance to nisin has been reported in rumen-specific *Streptococcus bovis* but in the same study nisin-resistant strains were still sensitive to monensin (Mantovani and Russell, 2001). Nisin resistance is also reported in *Streptomyces longisporoflavus,* most likely mediated by an efflux pump (Wong and Limbago, 2019). Moreover, narasin resistance has been associated with a plasmid-encoded resistance in *Enterococcus faecium* (Naemi et al., 2020). The plasmid-associated genes of resistance resulted in a decreased susceptibility to maduramicin, narasin and salinomycin but not to monensin. Importantly, narasin-resistant strains showed a high level of resistance to common antimicrobials used in human clinical medicine, including vancomycin. Salinomycin likely influences the expression of mobile resistance genes in *C. difficile* (Hosseinzadeh et al., 2016).

Earlier studies concluded that genes associated with resistance to ionophores are unlikely to be transferred to other bacterial species (Anon, 2007; Houlihan and Russell, 2003; Nisbet et al., 2008; Russell and Houlihan, 2003; Witte et al., 1999). However, resistance of any antimicrobial can be potentially associated with cross-resistance to other classes. Lately, in the case of ionophores, a few authors have reinitiated discussion regarding the likelihood of cross-resistance, particularly after the narasin- or tetranasin-related resistance was shown to be accompanied by a cross-resistance to vancomycin (Nilsson et al., 2012; Wong, 2019). In case of the narasin-related resistance to vancomycin, it decreased rapidly after discontinuation of the narasin-enriched diet for broilers (Simm et al., 2019). Additionally, in a small scale study, ionophore-mediated cross-resistance was demonstrated in *C. aminophilum* for bacitracin (Houlihan and Russell, 2003). Tetranasin resistance in *Prevotella ruminicola* (Newbold et al., 1988) is linked to low level resistance to avoparcin (Newbold et al., 1993), a glycopeptide that has been previously linked to a cross-resistance to vancomycin (Klare et al., 1995). Narasin resistance reported for poultry isolates of *E. faecium* is genetically coded by 6 known plasmids of which 4 are associated with vancomycin resistance determinants (Nilsson et al., 2016). Some of the genes of resistance were associated with changes in cell permeability and others with an ATP-dependent efflux pump (Linton et al., 1994; Nilsson et al., 2016). Indeed, resistance to one ionophore may result in a cross-resistance to other ionophores (Newbold et al., 1993; Nilsson et

al., 2016). However, the cross-resistance within ionophores has been detected only partially (Butaye et al., 2000, 2003). The presence of mobile genetic elements as determinants of antimicrobial resistance for some of the ionophores makes the danger of cross-resistance significantly increased (Frost et al., 2005).

Proposed measures to reduce the risk of development and spread of antimicrobial resistance arising from ionophore use into the future

A total ban on the use of antimicrobials in CAFOs is impractical particularly because of the beneficial effects to both animal and environmental health, and especially because of the lack of bona fide evidence of the impacts to human health from agricultural use of animal only antimicrobial agents such as ionophores. Nevertheless, it is important to note that sub-therapeutic administration to food-producing animals of all classes of antimicrobial used in human health has been banned throughout the world. Therapeutic, metaphylactic and prophylactic administration of antimicrobials will still be required in livestock, particularly for bacterial diseases not covered in their entirety by efficacious vaccines. There is currently no evidence that the use of ionophores (and in this particular case, monensin) as specified by the Australian feedlot cattle industry in accordance with the label instructions provided by the manufacturer results in any perceivable impact to human health, either through the direct consumption of meat or indirectly through environment contamination. Nevertheless, as ionophores are a class of naturally-occurring antibiotics and a transferrable plasmid-mediated narasin/salinomycin/maduramycin resistance mechanism (mediated by an ABC-like transporter) was recently described in poultry *E. faecium* (Naemi et al., 2020), we propose the following strategies to reduce the risk of monensin resistance arising in Australian beef feedlots in the future.

- Adhering to antimicrobial stewardship principles
    - Currently, ionophores are not listed as critically important, highly important or important antimicrobials by WHO and this is unlikely to change in the future
    - Using ionophores strictly in accordance with the label instructions as rumen modulators within an holistic animal management program that seeks to reduce overall use of antimicrobials with particular emphasis on critically important shared class drugs (e.g. ceftiofur).
- Maintaining animal health by using only credible alternatives to antimicrobial use
    - Administration of probiotics and prebiotics with proven efficacy claims in feedlot cattle
    - Improved overall management and stress reduction prior to, at entry and throughout the feed period
    - Use of immunisation to decrease the need for antimicrobials for particular diseases (e.g. vaccines against BRD)
    - Use of proven nutritional supplements to prevent rumen acidosis and bloat
- Controlled release of antimicrobials into the environment
    - Composting of all manure from beef feedlots would considerably decrease concentrations of ionophores (and other antimicrobials administered during confinement) in agricultural amendments. The composting period should be at least 80 days (4x the maximum t1/2)

    - Using alternative methods of manure decontamnation are also acceptable (e.g., anaerobic digestors; microbial incolulation; stock piling of manure for at least 12 weeks [4x the maximum t1/2]; or use of amendments in manure)
    - Preventing wastewater run offs and their entry into waterways
- Strategic surveillance of antimicrobial resistance determinants in commensal bacteria (e.g. Enterococcus) in healthy lotfed livestock.

Conclusion

It is clear from the literature reviewed that monensin, a naturally occurring ionophore that has a bacteriostatic mechanism of action against predominantly Gram-positive bacteria, performs a vital function in beef feedlots to modulate the rumen microbiota to improve animal health performance and reduce environmental impacts. To date, no resistance issues of concern to human health have been associated with monensin use in feedlot cattle, although a plasmid-mediated transferrable resistance mechanism against narasin (and some other ionophores, but not monensin) has recently been identified in *E. faecium* isolates from poultry in Scandinavia. As this resistance mechanism was co-located on a plasmid with VanA genes, cross-resistance is possible but appears to be negligible and of limited impact to human health. It is important to carry out regular surveys on the antimicrobial resistance patterns of *Enterococcus* spp. isolated from healthy feedlot cattle with the aim to detect mechanisms of resistance and to estimate risk to human clinical medicine, which is currently extremely low. In this case, monensin should be included as one of the antimicrobials in future antimicrobial susceptibility testing. Whole genome sequencing of isolates to identify potential resistance mechanisms and linkages with other genes on mobile genetic elements should be carried out on any ionophore-resistant strains identified in future surveillance programmes.

References

Aarestrup, F. M., Bager, F., Jensen, N. E., Madsen, M., Meyling, A., and Wegener, H. C. (1998). Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *APMIS : acta pathologica, microbiologica et immunologica Scandinavica* **106**, 606-622.

Abbott, L. K., Macdonald, L. M., Wong, M. T. F., Webb, M. J., Jenkins, S. N., and Farrell, M. (2018). Potential roles of biological amendments for profitable grain production – A review. *Agriculture, Ecosystems and Environment* **256**, 34-50.

Abreham, S., Teklu, A., Cox, E., and Sisay Tessema, T. (2019). Escherichia coli O157:H7: distribution, molecular characterization, antimicrobial resistance patterns and source of contamination of sheep and goat carcasses at an export abattoir, Mojdo, Ethiopia. *BMC Microbiol* **19**, 215.

Acar, J. F., and Moulin, G. (2006). Antimicrobial resistance at farm level. *OIE Revue Scientifique et Technique* **25**, 775-792.

Adator, E. H., Walker, M., Narvaez-Bravo, C., Zaheer, R., Goji, N., Cook, S. R., Tymensen, L., Hannon, S. J., Church, D., Booker, C. W., Amoako, K., Nadon, C. A., Read, R., and McAllister, T. A. (2020).

Whole genome sequencing differentiates presumptive extended spectrum beta-lactamase producing escherichia coli along segments of the one health continuum. *Microorganisms* **8**.

Agga, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M., and Schmidt, J. W. (2015). Antimicrobial-resistant bacterial populations and antimicrobial resistance genes obtained from environments impacted by livestock and municipal waste. *PLoS ONE* **10**.

Alexander, T. W., Yanke, L. J., Topp, E., Olson, M. E., Read, R. R., Morck, D. W., and McAllister, T. A. (2008). Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant Escherichia coli bacteria in feedlot cattle. *Applied and Environmental Microbiology* **74**, 4405-4416.

Amarakoon, I. D., Sura, S., Zvomuya, F., Cessna, A. J., Larney, F. J., and McAllister, T. A. (2016). Dissipation of antimicrobials in a seasonally frozen soil after beef cattle manure application. *Journal of Environmental Quality* **45**, 1644-1651.

Amarakoon, I. D., Zvomuya, F., Cessna, A. J., Degenhardt, D., Larney, F. J., and McAllister, T. A. (2014). Runoff losses of excreted chlortetracycline, sulfamethazine, and tylosin from surface-applied and soil-incorporated beef cattle feedlot manure. *Journal of Environmental Quality* **43**, 549-557.

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology* **8**, 260-271.

Angulo, F. J., Nargund, V. N., and Chiller, T. C. (2004). Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance. *J Vet Med B Infect Dis Vet Public Health* **51**, 374-9.

Anon (2007). Monensin (cattle, including dairy cows). (E. M. Agency, ed.), Vol. EMEA/CVMP/185123/2007-Final. EMEA Veterinary Medicines and Inspections.

Arias, C. A., Panesso, D., McGrath, D. M., Qin, X., Mojica, M. F., Miller, C., Diaz, L., Tran, T. T., Rincon, S., Barbu, E. M., Reyes, J., Roh, J. H., Lobos, E., Sodergren, E., Pasqualini, R., Arap, W., Quinn, J. P., Shamoo, Y., Murray, B. E., and Weinstock, G. M. (2011). Genetic Basis for In Vivo Daptomycin Resistance in Enterococci. *New England Journal of Medicine* **365**, 892-900.

Arikan, O. A., Mulbry, W., and Rice, C. (2009). Management of antibiotic residues from agricultural sources: Use of composting to reduce chlortetracycline residues in beef manure from treated animals. *Journal of Hazardous Materials* **164**, 483-489.

Arikan, O. A., Mulbry, W., and Rice, C. (2016). The effect of composting on the persistence of four ionophores in dairy manure and poultry litter. *Waste management (Elmsford)* **54**, 110-117.

Armstrong, J., and Spears, J. (1988). Intravenous administration of ionophores in ruminants: effects on metabolism independent of the rumen. *Journal of animal science* **66**, 1807-1817.

Azzaz, H. H., Murad, H. A., and Morsy, T. A. (2015). Utility of ionophores for ruminant animals: a review. *Asian Journal of Animal Sciences* **9**, 254-265.

Badger, S., Sullivan, K., Jordan, D., Caraguel, C., Page, S., Cusack, P., Frith, D., and Trott, D. (2020). Antimicrobial use and stewardship practices on Australian beef feedlots. *Australian Veterinary Journal* **98**, 37-47.

Bag, S., Ghosh, T. S., Banerjee, S., Mehta, O., Verma, J., Dayal, M., Desigamani, A., Kumar, P., Saha, B., Kedia, S., Ahuja, V., Ramamurthy, T., and Das, B. (2019). Molecular Insights into Antimicrobial Resistance Traits of Commensal Human Gut Microbiota. *Microb Ecol* **77**, 546-557.

Bailey, C., Spielmeyer, A., Hamscher, G., Schüttrumpf, H., and Frings, R. M. (2016). The veterinary antibiotic journey: comparing the behaviour of sulfadiazine, sulfamethazine, sulfamethoxazole and tetracycline in cow excrement and two soils. *Journal of Soils and Sediments* **16**, 1690-1704.

Barlow, R., Mcmillan, K., Mellor, G., Duffy, L., Jordan, D., Abraham, R., O'dea, M., Sahibzada, S., and Abraham, S. (2022). Phenotypic and Genotypic Assessment of Antimicrobial Resistance in Escherichia coli from Australian Cattle Populations at Slaughter. *Journal of Food Protection* **85**, 563-570.

Ben, Y., Fu, C., Hu, M., Liu, L., Wong, M. H., and Zheng, C. (2019). Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. *Environmental Research* **169**, 483-493.

Benchaar, C., Duynisveld, J., and Charmley, E. (2006). Effects of monensin and increasing dose levels of a mixture of essential oil compounds on intake, digestion and growth performance of beef cattle. *Canadian Journal of Animal Science* **86**, 91-96.

Bender, J. K., Cattoir, V., Hegstad, K., Sadowy, E., Coque, T. M., Westh, H., Hammerum, A. M., Schaffer, K., Burns, K., Murchan, S., Novais, C., Freitas, A. R., Peixe, L., Del Grosso, M., Pantosti, A., and Werner, G. (2018). Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resist Updat* **40**, 25-39.

Bergman, M., Nyberg, S. T., Huovinen, P., Paakkari, P., and Hakanen, A. J. (2009). Association between Antimicrobial Consumption and Resistance in Escherichia coli. *Antimicrobial Agents and Chemotherapy* **53**, 912-917.

Biavasco, F., Foglia, G., Paoletti, C., Zandri, G., Magi, G., Guaglianone, E., Sundsfjord, A., Pruzzo, C., Donelli, G., and Facinelli, B. (2007). VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* **73**, 3307-19.

Bohlmann, L., De Oliveira, D. M. P., El-Deeb, I. M., Brazel, E. B., Harbison-Price, N., Ong, C. L. Y., Rivera-Hernandez, T., Ferguson, S. A., Cork, A. J., Phan, M. D., Soderholm, A. T., Davies, M. R., Nimmo, G. R., Dougan, G., Schembri, M. A., Cook, G. M., McEwan, A. G., von Itzstein, M., McDevitt, C. A., and Walker, M. J. (2018). Chemical synergy between ionophore PBT2 and zinc reverses antibiotic resistance. *mBio* **9**.

Butaye, P., Devriese, L. A., and Haesebrouck, F. (2000). Incomplete cross resistance against ionophores in Enterococcus faecium and Enterococcus faecalis strains from pigs and poultry. *Microbial drug resistance (Larchmont, N.Y.)* **6**, 59-61.

Butaye, P., Devriese, L. A., and Haesebrouck, F. (2003). Antimicrobial Growth Promoters Used in Animal Feed: Effects of Less Well Known Antibiotics on Gram-Positive Bacteria. *Clinical Microbiology Reviews* **16**, 175-188.

Callaway, T. R., Adams, K. A., and Russell, J. B. (1999). The ability of "low G + C gram-positive" ruminal bacteria to resist monensin and counteract potassium depletion. *Curr Microbiol* **39**, 226-30.

Callaway, T. R., Edrington, T. S., Rychlik, J. L., Genovese, K. J., Poole, T. L., Jung, Y. S., Bischoff, K. M., Anderson, R. C., and Nisbet, D. J. (2003). Ionophores: Their use as ruminant growth promotants and impact on food safety. *Current Issues in Intestinal Microbiology* **4**, 43-51.

Callaway, T. R., and Russell, J. B. (2000). Variations in the Ability of Ruminal Gram-Negative Prevotella Species to Resist Monensin. *Current microbiology* **40**, 185-189.

Carlson, J. C., and Mabury, S. A. (2006). Dissipation kinetics and mobility of chlortetracycline, tylosin, and monensin in an agricultural soil in Northumberland County, Ontario, Canada. *Environmental Toxicology and Chemistry* **25**, 1-10.

Cessna, A. J., Larney, F. J., Kuchta, S. L., Hao, X., Entz, T., Topp, E., and McAllister, T. A. (2011). Veterinary antimicrobials in feedlot manure: Dissipation during composting and effects on composting processes. *Journal of Environmental Quality* **40**, 188-198.

Chen, B., Hao, L., Guo, X., Wang, N., and Ye, B. (2015). Prevalence of antibiotic resistance genes of wastewater and surface water in livestock farms of Jiangsu Province, China. *Environmental Science and Pollution Research* **22**, 13950-13959.

Chen, C., Ray, P., Knowlton, K. F., Pruden, A., and Xia, K. (2018). Effect of composting and soil type on dissipation of veterinary antibiotics in land-applied manures. *Chemosphere* **196**, 270-279.

Cheng, G., Ning, J., Ahmed, S., Huang, J., Ullah, R., An, B., Hao, H., Dai, M., Huang, L., Wang, X., and Yuan, Z. (2019a). Selection and dissemination of antimicrobial resistance in Agri-food production. *Antimicrobial Resistance and Infection Control* **8**.

Cheng, J. H., Tang, X. Y., and Cui, J. F. (2019b). Effect of long-term manure slurry application on the occurrence of antibiotic resistance genes in arable purple soil (entisol). *Science of the Total Environment* **647**, 853-861.

Cho, S., McMillan, E. A., Barrett, J. B., Hiott, L. M., Woodley, T. A., House, S. L., Frye, J. G., and Jackson, C. R. (2022). Distribution and Transfer of Plasmid Replicon Families among Multidrug-Resistant Enterococcus faecalis and Enterococcus faecium from Poultry. *Microorganisms* **10**.

Chow, J. M., and Russell, J. B. (1990). Effect of ionophores and pH on growth of Streptococcus bovis in batch and continuous culture. *Applied and Environmental Microbiology* **56**, 1588-1593.

Cingolani, V. (2021). Hypothesis of zinc ascorbate as best zinc ionophore for raising antiviral resistance against Covid-19. *Journal of medical virology*.

Cleary, D. W., Bishop, A. H., Zhang, L., Topp, E., Wellington, E. M. H., and Gaze, W. H. (2016). Long-term antibiotic exposure in soil is associated with changes in microbial community structure and prevalence of class 1 integrons. *FEMS Microbiology Ecology* **92**.

CLSI (2020). Performance Standards for Antimicrobial Susceptibility Testing. *In* "Clinical and Laboratory Standards Institute", Vol. 30th ed.

Cycoń, M., Mrozik, A., and Piotrowska-Seget, Z. (2019). Antibiotics in the soil environment—degradation and their impact on microbial activity and diversity. *Frontiers in Microbiology* **10**.

D'Alessio, M., Durso, L. M., Miller, D. N., Woodbury, B., Ray, C., and Snow, D. D. (2019). Environmental fate and microbial effects of monensin, lincomycin, and sulfamethazine residues in soil. *Environmental Pollution* **246**, 60-68.

Dawson, K. A., and Boling, J. A. (1984). Factors affecting resistance of monensin-resistant and sensitive strains of *Bacteroides ruminicola*. *Canadian Journal of Animal Science* **64**, 132-133.

De Oliveira, D. M. P., Bohlmann, L., Conroy, T., Jen, F. E. C., Everest-Dass, A., Hansford, K. A., Bolisetti, R., El-Deeb, I. M., Forde, B. M., Phan, M. D., Lacey, J. A., Tan, A., Rivera-Hernandez, T., Brouwer, S., Keller, N., Kidd, T. J., Cork, A. J., Bauer, M. J., Cook, G. M., Davies, M. R., Beatson, S. A., Paterson, D. L., McEwan, A. G., Li, J., Schembri, M. A., Blaskovich, M. A. T., Jennings, M. P., McDevitt, C. A., Von Itzstein, M., and Walker, M. J. (2020). Repurposing a neurodegenerative disease drug to treat Gram-negative antibiotic-resistant bacterial sepsis. *Science Translational Medicine* **12**.

Dennis, S., Nagaraja, T., and Bartley, E. (1981). Effect of lasalocid or monensin on lactate-producing or using rumen bacteria. *Journal of Animal Science* **52**, 418-426.

DeVries, S. L., and Zhang, P. (2016). Antibiotics and the Terrestrial Nitrogen Cycle: A Review. *Current Pollution Reports* **2**, 51-67.

Dolliver, H., Gupta, S., and Noll, S. (2008). Antibiotic degradation during manure composting. *Journal of Environmental Quality* **37**, 1245-1253.

Donoho, A., Manthey, J., Occolowitz, J., and Zornes, L. (1978). Metabolism of monensin in the steer and rat. *Journal of Agricultural and Food Chemistry* **26**, 1090-1095.

Du, L., and Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development* **32**, 309-327.

Durso, L. M., and Cook, K. L. (2014a). Impacts of antibiotic use in agriculture: what are the benefits and risks? *Curr Opin Microbiol* **19**, 37-44.

Durso, L. M., and Cook, K. L. (2014b). Impacts of antibiotic use in agriculture: What are the benefits and risks? *Current Opinion in Microbiology* **19**, 37-44.

Edrington, T. S., Looper, M. L., Duke, S. E., Callaway, T. R., Genovese, K. J., Anderson, R. C., and Nisbet, D. J. (2006). Effect of ionophore supplementation on the incidence of Escherichia coli O157:H7 and Salmonella and antimicrobial susceptibility of fecal coliforms in Stocker cattle. *Foodborne Pathog Dis* **3**, 284-91.

EFSA (2018). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA Journal* **16**, e05182.

Fauci, A. S., and Marston l, D. (2014). The perpetual challenge of antimicrobial resistance. *Jama* **311**, 1853-4.

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology* **3**, 722-732.

Gouliouris, T., Raven, K. E., Ludden, C., Blane, B., Corander, J., Horner, C. S., Hernandez-Garcia, J., Wood, P., Hadjirin, N. F., and Radakovic, M. (2018). Genomic surveillance of Enterococcus faecium reveals limited sharing of strains and resistance genes between livestock and humans in the United Kingdom. *MBio* **9**, e01780-18.

Granados-Chinchilla, F., Arias-Andrés, M. D. J., Fernández Montes de Oca, M. L., and Rodríguez, C. (2020). Effect of the veterinary ionophore monensin on the structure and activity of a tropical soil bacterial community. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes* **55**, 127-134.

Guan, H., Wittenberg, K. M., Ominski, K. H., and Krause, D. O. (2006). Efficacy of ionophores in cattle diets for mitigation of enteric methane. *Journal of Animal Science* **84**, 1896-1906.

Gurmessa, B., Pedretti, E. F., Cocco, S., Cardelli, V., and Corti, G. (2020). Manure anaerobic digestion effects and the role of pre- and post-treatments on veterinary antibiotics and antibiotic resistance genes removal efficiency. *Science of the Total Environment* **721**.

Hammerum, A. M. (2012). Enterococci of animal origin and their significance for public health. *Clinical Microbiology and Infection* **18**, 619-625.

Hao, H., Cheng, G., Iqbal, Z., Ai, X., Hussain, H. I., Huang, L., Dai, M., Wang, Y., Liu, Z., and Yuan, Z. (2014). Benefits and risks of antimicrobial use in food-producing animals. *Frontiers in Microbiology* **5**.

Harbison-Price, N., Ferguson, S. A., Heikal, A., Taiaroa, G., Hards, K., Nakatani, Y., Rennison, D., Brimble, M. A., El-Deeb, I. M., Bohlmann, L., McDevitt, C. A., von Itzstein, M., Walker, M. J., and Cook, G. M. (2020). Multiple bactericidal mechanisms of the zinc ionophore PBT2. *mSphere* **5**.

Herberg, R., Manthey, J., Richardson, L., Cooley, C., and Donoho, A. (1978). Excretion and tissue distribution of [14C]monensin in cattle. *Journal of Agricultural and Food Chemistry* **26**, 1087-1090.

Heuer, H., Schmitt, H., and Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Current Opinion in Microbiology* **14**, 236-243.

Ho, P. L., Ng, K. Y., Lo, W. U., Law, P. Y., Lai, E. L., Wang, Y., and Chow, K. H. (2016). Plasmid-Mediated OqxAB Is an Important Mechanism for Nitrofurantoin Resistance in Escherichia coli. *Antimicrob Agents Chemother* **60**, 537-43.

Hoogerheide, J. G., and Popov, A. I. (1979). A study of metal complexes of a naturally occurring macrocyclic ionophore-monensin. *Journal of Solution Chemistry* **8**, 83-95.

Hosseinzadeh, S., Mirsadeghi, E., Rajaian, H., Sayyadi, M., and Nazifi, S. (2016). Effect of ionophore salinomycin on the antibiotic resistance in Clostridium difficile detected in mice. *Comparative Clinical Pathology* **25**, 1137-1141.

Houlihan, A. J., and Russell, J. B. (2003). The susceptibility of ionophore-resistant Clostridium aminophilum F to other antibiotics. *Journal of Antimicrobial Chemotherapy* **52**, 623-628.

Hudson, J. A., Frewer, L. J., Jones, G., Brereton, P. A., Whittingham, M. J., and Stewart, G. (2017). The agri-food chain and antimicrobial resistance: A review. *Trends in Food Science and Technology* **69**, 131-147.

Jacobs, K., Wind, L., Krometis, L. A., Cully Hession, W., and Pruden, A. (2019). Fecal indicator bacteria and antibiotic resistance genes in storm runoff from dairy manure and compost-amended vegetable plots. *Journal of Environmental Quality* **48**, 1047-1056.

Jechalke, S., Heuer, H., Siemens, J., Amelung, W., and Smalla, K. (2014). Fate and effects of veterinary antibiotics in soil. *Trends in Microbiology* **22**, 536-545.

Jung, Y. H., Shin, E. S., Kim, O., Yoo, J. S., Lee, K. M., Yoo, J. I., Chung, G. T., and Lee, Y. S. (2010). Characterization of two newly identified genes, vgaD and vatH, [corrected] conferring resistance to streptogramin A in Enterococcus faecium. *Antimicrob Agents Chemother* **54**, 4744-9.

Kang, D. H., Gupta, S., Rosen, C., Fritz, V., Singh, A., Chander, Y., Murray, H., and Rohwer, C. (2013). Antibiotic Uptake by Vegetable Crops from Manure-Applied Soils. *Journal of Agricultural and Food Chemistry* **61**, 9992-10001.

Kaushik, V., Yakisich, J. S., Kumar, A., Azad, N., and Iyer, A. K. (2018). Ionophores: Potential use as anticancer drugs and chemosensitizers. *Cancers* **10**, 360.

Kemper, N. (2008). Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators* **8**, 1-13.

Kevin Ii, D. A., Meujo, D. A. F., and Hamann, M. T. (2009). Polyether ionophores: broad-spectrum and promising biologically active molecules for the control of drug-resistant bacteria and parasites. *Expert Opinion on Drug Discovery* **4**, 109-146.

Kim, K. R., Owens, G., Kwon, S. I., So, K. H., Lee, D. B., and Ok, Y. S. (2011). Occurrence and environmental fate of veterinary antibiotics in the terrestrial environment. *Water, Air, and Soil Pollution* **214**, 163-174.

Klare, I., Heier, H., Claus, H., Reissbrodt, R., and Witte, W. v. (1995). vanA-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry. *FEMS Microbiology Letters* **125**, 165-171.

Kuppusamy, S., Kakarla, D., Venkateswarlu, K., Megharaj, M., Yoon, Y. E., and Lee, Y. B. (2018). Veterinary antibiotics (VAs) contamination as a global agro-ecological issue: A critical view. *Agriculture, Ecosystems and Environment* **257**, 47-59.

Kyselková, M., Jirout, J., Chroňáková, A., Vrchotová, N., Bradley, R., Schmitt, H., and Elhottová, D. (2013). Cow excrements enhance the occurrence of tetracycline resistance genes in soil regardless of their oxytetracycline content. *Chemosphere* **93**, 2413-2418.

Kyselková, M., Kotrbová, L., Bhumibhamon, G., Chroňáková, A., Jirout, J., Vrchotová, N., Schmitt, H., and Elhottová, D. (2015). Tetracycline resistance genes persist in soil amended with cattle feces independently from chlortetracycline selection pressure. *Soil Biology and Biochemistry* **81**, 259-265.

Lana, R. P., Fox, D. G., Russell, J. B., and Perry, T. C. (1997). Influence of monensin on Holstein steers fed high-concentrate diets containing soybean meal or urea. *Journal of animal science* **75**, 2571-2579.

Lancaster, H., Roberts, A. P., Bedi, R., Wilson, M., and Mullany, P. (2004). Characterization of Tn916S, a Tn916-like element containing the tetracycline resistance determinant tet(S). *J Bacteriol* **186**, 4395-8.

Li, J., Wang, X., Cong, C., Wan, L., Xu, Y., Li, X., Hou, F., Wu, Y., and Wang, L. (2020). Inoculation of cattle manure with microbial agents increases efficiency and promotes maturity in composting. *3 Biotech* **10**.

Linton, K. J., Cooper, H. N., Hunter, L. S., and Leadlay, P. F. (1994). An ABC-transporter from Streptomyces longisporoflavus confers resistance to the polyether-ionophore antibiotic tetronasin. *Molecular Microbiology* **11**, 777-785.

Ma, Z., Lee, S., and Casey Jeong, K. (2019). Mitigating antibiotic resistance at the livestock-environment interface: A review. *Journal of Microbiology and Biotechnology* **29**, 1683-1692.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., and Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**, 268-81.

Mantovani, H. C., and Russell, J. B. (2001). Nisin resistance of Streptococcus bovis. *Applied and Environmental Microbiology* **67**, 808-813.

Marounek, M., and Rada, V. (1995). Susceptibility of Poultry Lactobacilli to Ionophore Antibiotics. *Journal of veterinary medicine. Series B* **42**, 193-196.

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clinical microbiology reviews* **24**, 718-733.

Mathers, J. J., Clark, S. R., Hausmann, D., Tillman, P., Benning, V. R., and Gordon, S. K. (2004). Inhibition of resistance plasmid transfer in Escherichia coli by ionophores, chlortetracycline, bacitracin, and ionophore/antimicrobial combinations. *Avian Dis* **48**, 317-23.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., and Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* **10**, 115-132.

McGarvey, J. A., Place, S., Palumbo, J., Hnasko, R., and Mitloehner, F. (2018). Dosage-dependent effects of monensin on the rumen microbiota of lactating dairy cattle. *Microbiologyopen* **8**, e783.

Melville, C. M., Brunel, R., Flint, H. J., and Scott, K. P. (2004). The Butyrivibrio fibrisolvens tet(W) gene is carried on the novel conjugative transposon TnB1230, which contains duplicated nitroreductase coding sequences. *J Bacteriol* **186**, 3656-9.

Muller, R. D., Potter, E. L., Wray, M. I., Richardson, L. F., and Grueter, H. P. (1986). Administration of monensin in self-fed (salt limiting) dry supplements or on an alternate-day feeding schedule. *J Anim Sci* **62**, 593-600.

Naemi, A.-O., Dey, H., Kiran, N., Sandvik, S. T., Slettemeås, J. S., Nesse, L. L., and Simm, R. (2020). NarAB Is an ABC-Type Transporter That Confers Resistance to the Polyether Ionophores Narasin, Salinomycin, and Maduramicin, but Not Monensin. *Frontiers in microbiology* **11**, 104-104.

Nagaraja, T. G., and Taylor, M. B. (1987). Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. *Appl Environ Microbiol* **53**, 1620-5.

NARMS (2011). Retail Meat Annual Report. *FDA*.

Netthisinghe, A. M. P., Cook, K. L., Gilfillen, R. A., Woosley, P. B., Kingery, T., and Sistani, K. R. (2018). Managing beef backgrounding residual soil contaminants by alum and biochar amendments. *Journal of Environmental Quality* **47**, 1275-1283.

Newbold, C. J., Wallace, R. J., and Walker, N. D. (1993). The effect of tetronasin and monensin on fermentation, microbial numbers and the development of ionophore-resistant bacteria in the rumen. *J Appl Bacteriol* **75**, 129-34.

Newbold, C. J., Wallace, R. J., Watt, N. D., and Richardson, A. J. (1988). Effect of the novel ionophore tetronasin (ICI 139603) on ruminal microorganisms. *Appl Environ Microbiol* **54**, 544-7.

Nilsson, O., Greko, C., Bengtsson, B., and Englund, S. (2012). Genetic diversity among VRE isolates from Swedish broilers with the coincidental finding of transferrable decreased susceptibility to narasin. *Journal of applied microbiology* **112**, 716-722.

Nilsson, O., Myrenås, M., and Ågren, J. (2016). Transferable genes putatively conferring elevated minimum inhibitory concentrations of narasin in Enterococcus faecium from Swedish broilers. *Veterinary microbiology* **184**, 80-83.

Nisbet, D. J., Callaway, T. R., Edrington, T. S., Anderson, R. C., and Poole, T. L. (2008). Effects of ionophores on Enterococcus faecalis and E. faecium growth in pure and mixed ruminal culture. *Foodborne Pathog Dis* **5**, 193-8.

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Cook, S. R., Zaheer, R., Yang, H., Woerner, D. R., Geornaras, I., McArt, J. A., Gow, S. P., Ruiz, J., Jones, K. L., Boucher, C. A., McAllister, T. A., Belk, K. E., and Morley, P. S. (2016a). Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Scientific Reports* **6**.

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Dettenwanger, A., Cook, S., Geornaras, I., Woerner, D. E., Gow, S. P., McAllister, T. A., Yang, H., Ruiz, J., Jones, K. L., Boucher, C. A., Morley, P. S., and Belk, K. E. (2016b). Resistome diversity in cattle and the environment decreases during beef production. *eLife* **5**.

Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., Gray, J. T., Kebreab, E., France, J., and McBride, B. W. (2007). Long-Term Effects of Feeding Monensin on Methane Production in Lactating Dairy Cows. *Journal of dairy science* **90**, 1781-1788.

Oliver, J. P., Gooch, C. A., Lansing, S., Schueler, J., Hurst, J. J., Sassoubre, L., Crossette, E. M., and Aga, D. S. (2020). Invited review: Fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. *Journal of Dairy Science* **103**, 1051-1071.

Oliver, W. (1975). Effect of monensin on gains of steers grazed on coastal bermudagrass. *Journal of Animal Science* **41**, 999-1001.

Overturf, M. D., Anderson, J. C., Pandelides, Z., Beyger, L., and Holdway, D. A. (2015). Pharmaceuticals and personal care products: A critical review of the impacts on fish reproduction. *Critical Reviews in Toxicology* **45**, 469-491.

Pan, M., and Chu, L. M. (2017). Fate of antibiotics in soil and their uptake by edible crops. *Science of the Total Environment* **599-600**, 500-512.

Place, S. E., Stackhouse, K. R., Wang, Q., and Mitloehner, F. M. (2011). Mitigation of greenhouse gas emissions from U.S. beef and dairy production systems. *In* "ACS Symposium Series", Vol. 1072, pp. 443-457.

Pokharel, S., Shrestha, P., and Adhikari, B. (2020). Antimicrobial use in food animals and human health: time to implement 'One Health' approach. *Antimicrobial Resistance & Infection Control* **9**, 181.

Pressman, B. C. (1976). Biological Applications of Ionophores. *Annual Review of Biochemistry* **45**, 501-530.

Pruden, A., Joakim Larsson, D. G., Amézquita, A., Collignon, P., Brandt, K. K., Graham, D. W., Lazorchak, J. M., Suzuki, S., Silley, P., Snape, J. R., Topp, E., Zhang, T., and Zhu, Y. G. (2013). Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives* **121**, 878-885.

Pu, C., Yu, Y., Diao, J., Gong, X., Li, J., and Sun, Y. (2019). Exploring the persistence and spreading of antibiotic resistance from manure to biocompost, soils and vegetables. *Science of the Total Environment* **688**, 262-269.

Qian, X., Gu, J., Sun, W., Wang, X. J., Su, J. Q., and Stedfeld, R. (2018). Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *Journal of Hazardous Materials* **344**, 716-722.

Quaik, S., Embrandiri, A., Ravindran, B., Hossain, K., Al-Dhabi, N. A., Arasu, M. V., Ignacimuthu, S., and Ismail, N. (2020). Veterinary antibiotics in animal manure and manure laden soil: Scenario and challenges in Asian countries. *Journal of King Saud University - Science* **32**, 1300-1305.

Ranga Niroshan Appuhamy, J. A. D., Strathe, A. B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., France, J., and Kebreab, E. (2013). Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. *Journal of dairy science* **96**, 5161-5173.

Ravva, S. V., Sarreal, C. Z., and Mandrell, R. E. (2013). Altered Protozoan and Bacterial Communities and Survival of Escherichia coli O157:H7 in Monensin-Treated Wastewater from a Dairy Lagoon. *PLoS ONE* **8**.

Ray, P., Chen, C., Knowlton, K. F., Pruden, A., and Xia, K. (2017). Fate and effect of antibiotics in beef and dairy manure during static and turned composting. *Journal of Environmental Quality* **46**, 45-54.

Rovira, P., McAllister, T., Lakin, S. M., Cook, S. R., Doster, E., Noyes, N. R., Weinroth, M. D., Yang, X., Parker, J. K., Boucher, C., Booker, C. W., Woerner, D. R., Belk, K. E., and Morley, P. S. (2019). Characterization of the Microbial Resistome in Conventional and "Raised Without Antibiotics" Beef and Dairy Production Systems. *Frontiers in Microbiology* **10**.

Russell, J. B., and Houlihan, A. J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiology Reviews* **27**, 65-74.

Ruuskanen, M., Muurinen, J., Meierjohan, A., Pärnänen, K., Tamminen, M., Lyra, C., Kronberg, L., and Virta, M. (2016). Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment. *Journal of Environmental Quality* **45**, 488-493.

Rychlik, J. L., and Russell, J. B. (2002). The adaptation and resistance of Clostridium aminophilum F to the butyrivibriocin-like substance of Butyrivibrio fibrisolvens JL5 and monensin. *FEMS microbiology letters* **209**, 93-98.

Sanderson, H., Fricker, C., Brown, R. S., Majury, A., and Liss, S. N. (2016). Antibiotic resistance genes as an emerging environmental contaminant. *Environmental Reviews* **24**, 205-218.

Sandoz, M. A., Wooten, K. J., Clendening, S. L., Hensley, L. L., Smith, L. R., and Smith, P. N. (2018). Transport mechanisms for veterinary pharmaceuticals from beef cattle feedyards to wetlands: Is aerial deposition a contributing source? *Agriculture, Ecosystems and Environment* **252**, 14-21.

Sassman, S. A., and Lee, L. S. (2007). Sorption and degradation in soils of veterinary ionophore antibiotics: monensin and lasalocid. *Environmental toxicology and chemistry* **26**, 1614-1621.

Schjørring, S., and Krogfelt, K. A. (2011). Assessment of bacterial antibiotic resistance transfer in the gut. *Int J Microbiol* **2011**, 312956.

Shakti, L., and Veeraraghavan, B. (2015). Advantage and limitations of nitrofurantoin in multi-drug resistant Indian scenario. *Indian J Med Microbiol* **33**, 477-81.

Shen, Y., Stedtfeld, R. D., Guo, X., Bhalsod, G. D., Jeon, S., Tiedje, J. M., Li, H., and Zhang, W. (2019). Pharmaceutical exposure changed antibiotic resistance genes and bacterial communities in soil-surface- and overhead-irrigated greenhouse lettuce. *Environment International* **131**.

Sigle, H. C., Schäfer-Korting, M., Korting, H. C., Hube, B., and Niewerth, M. (2006). In vitro investigations on the mode of action of the hydroxypyridone antimycotics rilopirox and piroctone on Candida albicans. *Mycoses* **49**, 159-168.

Simjee, S., Heffron, A.-L., Pridmore, A., and Shryock, T. R. (2012). Reversible monensin adaptation in Enterococcus faecium, Enterococcus faecalis and Clostridium perfringens of cattle origin: potential impact on human food safety. *Journal of Antimicrobial Chemotherapy* **67**, 2388-2395.

Simm, R., Slettemeås, J. S., Norström, M., Dean, K. R., Kaldhusdal, M., and Urdahl, A. M. (2019). Significant reduction of vancomycin resistant E. faecium in the Norwegian broiler population coincided with measures taken by the broiler industry to reduce antimicrobial resistant bacteria. *PloS one* **14**, e0226101-e0226101.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Herman, L., Haesebrouck, F., and Butaye, P. (2010). Broad-spectrum β-lactamases among Enterobacteriaceae of animal origin: molecular aspects, mobility and impact on public health. *FEMS microbiology reviews* **34**, 295-316.

Spielmeyer, A. (2018). Occurrence and fate of antibiotics in manure during manure treatments: A short review. *Sustainable Chemistry and Pharmacy* **9**, 76-86.

Storteboom, H. N., Kim, S. C., Doesken, K. C., Carlson, K. H., Davis, J. G., and Pruden, A. (2007). Response of antibiotics and resistance genes to high-intensity and low-intensity manure management. *Journal of Environmental Quality* **36**, 1695-1703.

Tangcharoensathien, V., Chanvatik, S., and Sommanustweechai, A. (2018). Complex determinants of inappropriate use of antibiotics. *Bulletin of the World Health Organization* **96**, 141-144.

Tasho, R. P., and Cho, J. Y. (2016). Veterinary antibiotics in animal waste, its distribution in soil and uptake by plants: A review. *Science of the Total Environment* **563-564**, 366-376.

Tedeschi, L. O., Fox, D. G., and Tylutki, T. P. (2003). Potential environmental benefits of ionophores in ruminant diets. *Journal of Environmental Quality* **32**, 1591-1602.

Toth, J. D., Feng, Y., and Dou, Z. (2011). Veterinary antibiotics at environmentally relevant concentrations inhibit soil iron reduction and nitrification. *Soil Biology and Biochemistry* **43**, 2470-2472.

Tripathi, V., and Tripathi, P. (2017). Antibiotic resistance genes: An emerging environmental pollutant. *In* "Environmental Science and Engineering (Subseries: Environmental Science)", pp. 183-201.

Tyrrell, C., Burgess, C. M., Brennan, F. P., and Walsh, F. (2019). Antibiotic resistance in grass and soil. *Biochemical Society Transactions* **47**, 477-486.

Udikovic-Kolic, N., Wichmann, F., Broderick, N. A., and Handelsman, J. (2014). Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proceedings of the National Academy of Sciences* **111**, 15202-15207.

Vertesy, L., Heil, K., Fehlhaber, H.-W., and ZIEGLER, W. (1987). Microbial decomposition of salinomycin. *The Journal of antibiotics* **40**, 388-390.

Vikram, A., Rovira, P., Agga, G. E., Arthur, T. M., Bosilevac, J. M., Wheeler, T. L., Morley, P. S., Belk, K. E., and Schmidt, J. W. (2017). Impact of "Raised without Antibiotics" Beef Cattle Production Practices on Occurrences of Antimicrobial Resistance. *Appl Environ Microbiol* **83**.

Walczak, J. J., and Xu, S. (2011). Manure as a source of antibiotic-resistant Escherichia coli and enterococci: A case study of a Wisconsin, USA family dairy farm. *Water, Air, and Soil Pollution* **219**, 579-589.

Watanabe, K., Watanabe, J., Kuramitsu, S., and Maruyama, H. B. (1981). Comparison of the activity of ionophores with other antibacterial agents against anaerobes. *Antimicrobial Agents and Chemotherapy* **19**, 519-525.

Watanabe, N., Bergamaschi, B. A., Loftin, K. A., Meyer, M. T., and Harter, T. (2010). Use and environmental occurrence of antibiotics in freestall dairy farms with manured forage fields. *Environmental Science and Technology* **44**, 6591-6600.

Watanabe, N., Harter, T. H., and Bergamaschi, B. A. (2008). Environmental Occurrence and Shallow Ground Water Detection of the Antibiotic Monensin from Dairy Farms. *Journal of environmental quality* **37**, S-78-S-85.

Webb, H. E., Angulo, F. J., Granier, S. A., Scott, H. M., and Loneragan, G. H. (2017). Illustrative examples of probable transfer of resistance determinants from food animals to humans: Streptothricins, glycopeptides, and colistin. *F1000Res* **6**, 1805.

WHO (2015). Global action plan on antimicrobial resistance.

Wildenauer, F. X., Blotevogel, K. H., and Winter, J. (1984). Effect of monensin and 2-bromoethanesulfonic acid on fatty acid metabolism and methane production from cattle manure. *Applied Microbiology and Biotechnology* **19**, 125-130.

Winkworth, C. L., and Lear, G. (2014). Antimicrobial potential of the ionophore monensin on freshwater biofilm bacteria. *Environ Sci Pollut Res Int* **21**, 10139-50.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science* **279**, 996-7.

Witte, W., Klare, I., and Werner, G. (1999). Selective pressure by antibiotics as feed additives. *Infection* **27 Suppl 2**, S35-8.

Wong, A. (2019). Unknown Risk on the Farm: Does Agricultural Use of Ionophores Contribute to the Burden of Antimicrobial Resistance? *Msphere* **4**, e00433-19.

Wong, A., and Limbago, B. M. (2019). Unknown Risk on the Farm: Does Agricultural Use of Ionophores Contribute to the Burden of Antimicrobial Resistance? *mSphere* **4**, e00433-19.

Wu, N., Zhang, W., Xie, S., Zeng, M., Liu, H., Yang, J., Liu, X., and Yang, F. (2020). Increasing prevalence of antibiotic resistance genes in manured agricultural soils in northern China. *Frontiers of Environmental Science and Engineering* **14**.

Yoshida, N., Castro, M. J. L., and Cirelli, A. F. (2010). Degradation of monensin on soils: influence of organic matter and water content. *Chemistry in ecology* **26**, 27-33.

Youngquist, C. P., Mitchell, S. M., and Cogger, C. G. (2016). Fate of antibiotics and antibiotic resistance during digestion and composting: A review. *Journal of Environmental Quality* **45**, 537-545.