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An investigation of factors influencing antimicrobial resistance in dairy herds

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Abstract

The emergence of antimicrobial resistance (AMR) as a result of the selective pressures placed on both commensal and pathogenic bacterial populations as a result of overuse and misuse of antimicrobials is one of the greatest issues facing human healthcare. Antimicrobials are widely used in agriculture for the maintenance of health and welfare, but their use also contributes to the issue of AMR and poses a risk to human health via the food chain. In order to tackle the challenge, governments and organisations across the world have committed to reducing antimicrobial use (AMU) in agriculture and to implement surveillance programmes to monitor AMR. Although AMU in agriculture in the United Kingdom (UK) is reducing, there remains a knowledge gap regarding the dynamics which exist in terms of AMU/AMR associations and the influences of the wider farm environment.

This context provided the rationale behind the research carried out and presented in this thesis. Chapter 1 provides an overview of available literature to explore the context and an outline of research aims.

In Chapter 2, a study group of sixteen dairy farms were recruited to investigate the associations between historical trends of AMU and AMR as part of a longitudinal study. AMU was determined over the course of six years and AMR was measured according to the minimum inhibitory concentration of sentinel bacterial species isolates from bulk tank milk samples. The findings of this Chapter demonstrated that higher levels of AMU did not necessarily represent higher levels of resistance and led to an interest in other influencing factors.

Chapter 3 outlines a cross sectional study investigating the influences of farm management practices on levels of resistance on dairy farms. Data was sourced from two study groups, one of which represented the herds recruited in Chapter 2, and utilised questionnaire responses collected during farm visits. Data was analysed using a robust modelling procedure and highlighted a range of management procedures existing across the dairy farm which may be associated with levels of resistance in sentinel bacteria.

Chapters 4 and 5 sought to outline a new laboratory based methodology which could be employed in the monitoring of on farm AMR via sampling of bulk tank milk. Initial investigations took the form of a pilot study, in which raw bulk tank milk samples were enriched using selective nutrient broths. The results of these initial investigations helped to inform a potential antimicrobial susceptibility testing (AST) methodology. This was further investigated with validation to compare experimental methodology with already established testing standards. Comparisons of the final methodology with validation steps demonstrated viability of the AST method.

Investigations of AMU/AMR interactions were once again considered in Chapter 6. AMU data, collated from farm medicine use records, were obtained from farms where bulk tank milk samples were sourced as part of investigations in Chapter 5. Analysis indicated that where statistically significant relationships between AMU and AMR existed, these relationships were negatively correlated.

Together, the findings of each of the Chapters presented in this thesis help to further our knowledge of the dynamics which exist with regards to AMR in the dairy farm setting, and provide an opportunity to further develop AMR surveillance across the industry.

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List of Abbreviations

AMR	Antimicrobial resistance
AMU	Antimicrobial use
AST	Antimicrobial susceptibility testing
AZ	Azide Dextrose (broth)
BMD	Broth microdilution
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DANMAP	The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
DCD	Defined Course Dose
DDD	Defined Daily Dose
EE	Enterobacteriacae Enrichment (broth)
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organisation
MALDI TOF-MS	Matrix-Assisted Laser Desorption Ionization/Time of Flight Mass Spectrometry
MIC	Minimum inhibitory concentration
OIE	Office International des Epizooties (World Organisation for Animal Health Office)
PBP	Penicillin binding proteins
RUMA	Responsible Use of Medicines in Agriculture Alliance
SB	Slanetz and Bartley (Agar)
TBX	Tryptone Bile X-Glucuronide (Agar)
UK	United Kingdom
VARSS	Veterinary Antimicrobial Resistance and Sales Surveillance
VMD	Veterinary Medicines Directorate
VRBG	Violet Red Bile Glucose (Agar)
WHO	World Health Organisation

Publications

Chapter 3; the work in this chapter was submitted to the Journal *Preventive Veterinary Medicine*

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Ethical approval

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

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Chapter 1. Introduction and literature review

In this first chapter, an introduction to antimicrobials – a brief history, discovery, their importance and mechanisms of action is discussed. The emergence of antimicrobial resistance (AMR) and the subsequent issues this presents is explored, along with an elaboration on current literature regarding antimicrobial use (AMU) and AMR in livestock agriculture, with specific attention to the dairy industry. This chapter will also address the aims and objectives of this PhD thesis.

1.1 Introduction to antimicrobials

1.1.1 Brief history

Treatments for diseases of bacterial origin exploiting the antimicrobial properties of organic materials has existed in human society for millennia, dating back to the times of ancient Greece and Egypt (Hutchings et al., 2019). Antimicrobial compounds are active against microorganisms (bacteria, fungi, viruses or protozoa) and can either kill or inhibit growth and multiplication. Within this umbrella term exists antibiotics, which are specifically active against bacteria (American Veterinary Medical Association, 2022). In scientific literature, antibiotics are often referred to as antimicrobials. For the purposes of this thesis, the term antimicrobial will be used when referring to antibiotics.

The first notable use of an antimicrobial effective against bacteria occurred at the turn of the twentieth century following the isolation of what would be called pycocyanase from *Pseudomonas aeruginosa* by the German scientists Emmerich and Low (Arbab et al., 2022). However, it was Sir Alexander Fleming's accidental discovery of penicillin, an unstable compound isolated from the fungus *Penicillium notatum*, which heralded a new age in the fight against bacterial infections (Kardos and Demain, 2011). A significant period of discovery and isolation of antimicrobial compounds derived from bacterial, fungal and synthetic sources across the two decades between 1940 and 1960 occurred. This period is sometimes referred to as a

'golden age' of antimicrobial discovery, which was followed by a period of chemical refinement of these compounds (Walsh and Wencewicz, 2014). The initial success of antimicrobials led them to be thought of as the "ultimate cure" (Penesyan et al., 2015). Wise (2002) argues that in the last 50 years, antimicrobial agents have improved public health to a greater extent than any other medical or scientific measure.

1.1.2 How do antimicrobials work?

The mediation of cell death as a result of antimicrobial action features a series of complex biological processes which are characterised by interactions between the antimicrobial agent and the target cell and the subsequent structural, molecular and biochemical modifications which follow (Kohanski et al., 2010). Antimicrobial agents are tested *in vitro* to ascertain their capacity to not only inhibit bacterial growth and division, but also the ability of the agent to eradicate the bacterial population from the site of infection. The former of these modalities is termed "bacteriostatic" and the latter "bactericidal" (Pankey and Sabath, 2004). Antimicrobial drugs used in the treatment of bacterial infections can be defined by their mechanisms of action, of which there are generally four. They may; inhibit metabolic pathways, inhibit protein synthesis, interrupt nucleic acid synthesis or hinder cell wall synthesis (Tenover, 2006). **Error! Reference source not found.** outlines some key concepts with respect t o antimicrobial function.

There are a number of factors which influence the response of an antimicrobial when being tested *in vitro* under strict laboratory conditions, such as growth conditions and the length of time antimicrobial action is tested for. These are further complicated *in vivo* due to the availability of nutrients for bacterial growth in the host as well as differences in host characteristics (Cozens et al., 1986). As a result, it has been stated that it is difficult to be able to ascertain the mechanisms of action of an antimicrobial agent in a clinically meaningful way (Nemeth et al., 2015). Therefore, it is often recommended that most antimicrobials be characterised as having both

bacteriostatic and bactericidal properties, in order to achieve the best cure rates possible (Pankey and Sabath, 2004).

Table 1.1. The effect of different classes of antimicrobials on bacteria and how they function i.e. bacteriostatic or bactericidal, and examples of individual drugs within these classes (adapted from Hooper, 2001 and Nemeth et al., 2015).

Effect on Bacterial Target	Class of Antimicrobial	Function	Example Drug
Inhibition of metabolic pathways	Sulfonamides Trimethoprim	both bacteriostatic	Sulfamethoxazole
Inhibition of protein synthesis	Aminoglycosides Chloramphenicol	bactericidal	Gentamicin
	Streptogramins	bacteriostatic or bactericidal	Pristinamycin Tetracylin, Doxycycline Linezolid Lincomycin
	Tetracyclines Oxazolidinones Lincosamides Macrolides	bacteriostatic	Erythromycin
Inhibition of nucleic acid synthesis	Fluoroquinolones Aminocoumarins	bactericidal bacteriostatic	Ciprofloxacilin Coumermycin
Disruption of cell wall integrity	β-Lactams Glycopeptides	both bactericidal	Penicillins, Cephalosporins Vancomycin

1.1.2.1 Inhibition of metabolic pathways

When considering the range of antimicrobial effects as outlined in Table 1.1, let us first consider how the metabolic pathways of the bacterial cell can be disrupted. Perhaps the most widely recognised biosynthetic bacterial pathway which is targeted by antimicrobial agents is that of the folic acid pathway within the cell. Both prokaryotic and eukaryotic cells require folate in order to produce molecules necessary in nucleic acid synthesis. Bacteria synthesise their own folate, and the required biosynthetic pathway makes this a possible antimicrobial drug target. The resulting impact is the disruption of nucleic acid synthesis of microorganisms, without affecting the cells of the host (Bermingham and Derrick, 2002). Disruption of folate biosynthesis occurs by the action of two agents, sulfamethoxazole (a sulfonamide) and trimethoprim, which target two steps in the biosynthesis of folate. Within this

pathway, the sulfamethoxazole competitively inhibits dihydropteroate synthase (DHPS) and trimethoprim (an example of a diaminopyrimidine) competitively inhibits dihydrofolate reductase (DHFR) (Projan, 2002). DHPS is an enzyme critical for dihydropteroic acid production from para-aminobenzoic acid (pABA) and pteridine, which is converted into dihydrofolate. Dihydrofolate is then converted to tetrahydrofolate by the action of the DHFR enzyme. It is this tetrahydrofolate which is essential in the synthesis of purines and pyrimidines for nucleic acid production (Capasso and Supuran, 2014). The combination of these two agents allows for synergistic inhibition of bacterial growth, and this combination is highly effective in the treatment of infectious conditions such as upper respiratory tract infections, urinary tract infections and bacterial endocarditis (Schiffman, 1975).

1.1.2.2 Inhibition of protein synthesis

Inhibition of the synthesis of proteins within the bacterial cell is a second method of antibacterial action. This is an available option due to the unique nature of the protein and RNA machinery within the prokaryotic cell. Protein synthesis is a complex, multi-step process which involves the interaction of molecules in initiation, elongation and termination of protein assembly, representing a number of intermediary steps which can be targeted by the action of antimicrobial molecules (Walsh, 2000). In protein synthesis, the genetic material of the bacteria (DNA) is transcribed to RNA via RNA polymerase which is known as messenger RNA (mRNA). This encodes the protein sequence, and the decoding of this and the subsequent assembly of amino acids into a protein sequence is known as translation (which occurs with the action of ribosomes) (Walsh and Wencewicz, 2014). Antimicrobials targeting such pathways often interfere with the action of bacterial ribosomes, with the prokaryotic ribosome consisting of the larger 50S subunit which are targeted for example by macrolides or streptogramins, and the smaller 30S subunit which are targeted by tetracyclines and aminoglycosides (Tenover, 2006).

Concerning aminoglycosides, these antimicrobials cause electrostatic interactions with core linkages in the 16S rRNA portion of the 30S ribosomal subunit. This action

is brought about by the 2-deoxystreptamine core sugar which is characteristic of the aminoglycoside family of antimicrobials. The consequence of such interaction between the active portion of the agent and the ribosome is to increase the incidence of errors in translation thus allowing for multiple mistakes per protein during, which can have lethal effects for the bacteria (Tenson and Mankin, 2006). Tetracyclines on the other hand inhibit bacterial protein synthesis by countering the binding of the aminoacyl-tRNA with the 30S subunit. The disruption of such binding by tetracyclines results in the translation step, required for the assembly of a protein sequence of mRNA, to be blocked. This prevents the sequencing of proteins important for the bacteria to grow and reproduce (Chopra and Roberts, 2001).

When considering antimicrobial action on the 50S ribosomal subunit, there are a wide variety of agents available which affect this, such as macrolides and the streptogrammins. Macrolides for example, specifically bind with the 23S rRNA of the large subunit. The net overall effect of macrolide action is that there is a resulting accumulation of peptidyl-tRNA, indicating that protein sequencing was being completed prematurely as a result of peptidyl tRNA disassociation. As a result, incomplete proteins are synthesised, resulting in inhibition of growth (Katz and Ashley, 2005). Streptogramins work on a similar basis. The streptogramin class consists of two subgroups; A and B, but are produced simultaneously, and work synergistically with both subgroups binding with the P site in the 23S rRNA (as with macrolides). Synergistic activity yields a net bactericidal effect whereas when they work independently the overall effect is primarily bacteriostatic. Subgroup A functions by blocking substrate attachment, hindering elongation of the protein chain. Additionally, this binding causes conformational change of the 50S ribosome, allowing for increased activity of subgroup B. The actions of the B subgroup are; inhibition of protein elongation and cause premature protein formation (resulting in non-functional peptides). They are also able to bind to the ribosomes at any stage of the synthesis process, which can result in major disruption of synthesis (Mukhtar and Wright, 2005). Therefore, the modalities of these antimicrobial agents bring about major disruption in protein synthesis, resulting in inhibition of growth, and when in high enough concentrations, can bring about cell death.

1.1.2.3 Inhibition of nucleic acid synthesis

Another prime target for antimicrobial agents is the targeting and disruption of the synthesis of nucleic acids, the genetic material of the bacteria. The primary example for this modality are the fluoroquinolones, with the major targets of antimicrobials of this class being the type II topoisomerase enzymes, of which bacteria have two related subsets; DNA gyrase and topoisomerase IV (these are found only in bacterial cells) (Walsh and Wencewicz, 2017). Topoisomerase enzymes are necessary for the separating of the bacterial DNA strands, formation of a complementary DNA strand from the original and subsequent annealing of the newly formed DNA strand. DNA gyrase forms negative superhelical twists into the DNA, which are a necessary requirement to allow for DNA replication and erase positive superhelical twists. The action of topoisomerase IV is largely in the closing stages of replication, causing separation of daughter chromosomes formed during the replication process, allowing for segregation into daughter cells, which would normally allow for bacterial replication (Hooper, 2000). Fluoroquinolones act by interrupting the enzyme-bound DNA complex (how bacterial DNA interacts with either the DNA gyrase or topoisomerase IV enzymes). The antimicrobial agent will incorporate itself into this complex, forming a new drug-enzyme-DNA complex. This however prevents the replication fork from proceeding in order to complete the replication cycle. This ultimately leads to inhibition of routine DNA replication and synthesis in the bacterial cell, resulting in cell death (Blondeau, 2004).

1.1.2.4 Disrupting bacterial cell wall integrity

In addition to the three methods described above, targeting of the bacterial cell is arguably the most widely preferred method to have been taken advantage of in the development of antimicrobial agents (Hooper, 2001). The cell walls of bacteria are essential for survival, as they help to give structure and prevent cell death due to osmotic interactions between the bacteria and its environment. The bacterial cell wall is made up of a series of peptidoglycan layers, and it is this layer along with the components necessary for synthesis of these layers that are targeted by the clinically

important antimicrobial classes of β-lactams and glycopeptides (Yount and Yeaman, 2013). β-lactams, named for their β-lactam ring, target penicillin-binding proteins (PBPs), enzymes which are involved in the restructuring and biosynthesis of the bacterial cell wall, and therefore, the crucial peptidoglycan structure. The β-lactam ring is complementary to the structure of D-alanine-D-alanine terminals, which form the building blocks of the peptidoglycan layer, and interact with the enzymes D,D-transpeptidase and D,D-carboxypeptidases (both are PBPs) which would normally allow for new layers of peptidoglycan to be synthesised and laid down. However, the binding of the β-lactam ring, which is an analogue of the D-ala-D-ala terminal, to the active sites of the PBPs, causes long term intermediate acylation, causing active sites to be sterically blocked for further acyl transfer. The result of such action is the inhibition of the PBPs, meaning that final crosslinking of peptidoglycan cannot occur. This interference with cell wall synthesis causes accumulation of peptidoglycan precursors whose presence triggers breakdown of the current peptidoglycan layer via autolytic hydrolysis. This, combined with the lack of new peptidoglycan production, brings about bacterial osmotic lysis, and therefore death of the cell (Llarrull et al., 2010; Schneider and Sahl, 2010). Similarly, glycopeptides, such as vancomycin, work by interfering with the bacterial cell wall. They too target the D-alanine-D-alanine terminal, preventing its binding with the PBPs thus inhibiting peptidoglycan cross linking, weakening the cell structure and causing bacterial cell death as a result of excessive osmotic pressures (Kang and Park, 2015).

1.2 Antimicrobial resistance

The status of antimicrobials as revolutionary, life saving medicines cannot be understated. However, the emergence of resistance by bacteria towards these drugs, and indeed increasing levels of such resistance, has jeopardised their capability to effectively combat bacterial infections, bringing about a rise in mortality from such infections, which were once readily curable (Collignon et al., 2016). The materialisation of the problem of AMR poses a considerable threat to the health status of the global population and has the potential to place a considerable burden on global health services (Högberg et al., 2010). The scale of AMR has received high

levels of attention in past decades, and this can easily be identified from the breadth of published literature available and increasing media coverage.

The World Health Organisation (WHO) defines AMR as occurring when "microorganisms such as bacteria, viruses, fungi and parasites change in ways that render the medications used to cure the infections they cause ineffective" (World Health Organisation, 2017). The emergence of AMR threatens to interfere with the headway made in the effort to control bacterial infections. As of 2016, it was estimated that resistance to antimicrobial drugs was associated with an estimated 23,000 deaths in the United States and 25,000 deaths in Europe per year. It was also estimated that the financial impact in the United States was around $20 billion in excess medical spending per year (Marston et al., 2016). A UK parliamentary commission report on AMR (known as the O'Neill report) published in 2016 predicted that by 2050, 10 million lives a year and up to $100 trillion of economic productivity are at risk due to the developing issue that is antimicrobial resistance (O'Neill, 2016).

Since the initial introduction of antimicrobials in medicine, there were warnings that the pathogens they were designed to eliminate would gain the ability to resist them and acquire defences to protect themselves (Chioro et al., 2015). In fact, it was as early as 1945, just soon after the introduction of penicillin, that Sir Alexander Fleming warned that the dangers of misuse and overuse of these antimicrobial agents would lead to them becoming ineffective. By 1948, it was calculated that the level of resistance among strains of *Staphylococcus aureus* in a London hospital was 38%. By 2013, it was estimated that 90% of *S. aureus* strains in the UK were resistant to penicillin (Huttner et al., 2013).

1.2.1 How do bacteria become resistant?

The mechanisms of antimicrobial action have been previously considered, as well an overview of the scale of the problem of AMR for human medicine. We now consider why and how bacteria become resistant to antimicrobial agents.

The increasing incidence of resistance over the past number of decades has come about through the action of evolution and the principles laid out by Charles Darwin of natural selection and 'survival of the fittest'. When considering a large population of bacteria, some of them will possess advantageous characteristics which allow them to survive when exposed to unfavourable conditions, for example through the application of an antimicrobial agent. While the majority of the bacterial population will be killed off as a result, those individuals with the favourable characteristics, which allows them to survive, will be passed on to the next generation of bacterial daughter cells or passed on to other bacteria in the environment. The phenotypes of bacteria displaying such characteristics are obtained by the attainment of resistance genes (White and McDermott, 2001). Recent research has found that resistance genes were present in organisms isolated from 30,000 year old permafrost deposits from Alaska. From their work, the researchers concluded that the emergence of AMR predates their use in modern medicinal practice and that resistance is ultimately going to occur in the environment (D'Costa et al., 2011). Due to the fact that most of the first antimicrobials to be isolated were obtained from bacteria and fungi, they must therefore have mechanisms in place to protect themselves from the actions of the agents they produce. Interestingly, such determinants of resistance have been found to share similarity with those found in resistant bacterial isolates in clinical environments (Webb and Davies, 1993). Despite resistance to antimicrobials having been shown to be naturally occurring in bacterial populations, the current crisis of AMR has come about due to the wide scale use of these agents in quantities never seen before. Global usage of antimicrobials was estimated to be in excess of one million tonnes since their introduction in medicine in the 1940s with a 2002 estimate putting global annual usage at between 100,000 and 200,000 tonnes (Andersson and Hughes, 2010).

1.2.1.1 Targeting of antimicrobial molecules

One of the most important mechanisms of bacterial resistance to antimicrobial molecules is by interfering with the molecule either through modification or destruction. Chemical modification is brought about by the production of enzymes

which can cause acetylation, phosphorylation, or adenylation. Acetylation has been shown to affect aminoglycosides and streptogrammins; phosphorylation affects aminoglycosides and chloramphenicol while adenylation affects aminoglycosides and lincosamides. The result of these modifications is a reduced affinity of the antimicrobial for its bacterial target. Alternatively, bacteria may be able to produce enzymes which destroy the antimicrobial agent altogether with the most well known of these being β-lactamases. These disrupt the amide bonding within the β-lactam ring via hydrolysis, resulting in the antimicrobial losing all effectiveness due to loss of conformation, in which affinity for its bacterial target is lost (Munita and Arias, 2016).

1.2.1.2 Interruption of antimicrobial target

A second method through which bacterial resistance can be conveyed is by interrupting the antimicrobial's access to its target. One such method to achieve this is for advantageous mutations within the bacteria to cause the downregulation of non-selective membrane channel proteins and upregulation of more highly selective channels. This therefore reduces the permeability of the bacterial outer membrane, thus limiting antimicrobial entry to the bacterium, which is a major characteristic of resistance in Enterobacteriaceae. Interrupting antimicrobial delivery into the bacterium can also be achieved through the function of efflux pumps. Bacterial efflux pumps allow antimicrobial compounds to be actively transported out of the cell, with these pumps ranging from highly selective to highly non-selective, allowing for considerable range in resistance capability. Non selective pumps therefore are sometimes referred to as multidrug resistance efflux pumps (Poole, 2005).

1.2.1.3 Modification of bacterial target

Additionally, bacteria can gain resistance as a result of molecular changes and modification to the target of antimicrobial molecules within the bacterial cell. Point mutations can cause changes in the target structure which can interfere with binding between it and the antimicrobial agent. At first this may seem detrimental to

necessary functions within the bacterium, but it has been shown that such mutations allow for the bacterial target to function naturally within the cell (Wright, 2011). Similarly, changes can be made which allow the bacterial targets to become protected from antimicrobials without the need for mutational alterations. One example is the methylation of 16S rRNA by erythromycin ribosome methylase. This methylation inhibits the binding of a number of antimicrobial classes, such as macrolides and streptogrammins, from binding, thus preventing them from bringing about bacterial cell death as a result of growth inhibition due to interrupted protein synthesis (Blair et al., 2015).

1.3 Antimicrobial use in agriculture

Given the projected impact AMR will have on the global population both for human health and economics in the coming decades, it is not surprising that the conversation around it is largely concentrated on the consequences for human health. Nevertheless, in addition to the losses facing the global population as a result of the potential prevalence of AMR in human medicine, a considerable impact could also be seen in the field of veterinary medicine if levels of resistance were to reach a similarly critical level. This would lead to significant economic losses as well as reductions in levels of animal welfare (Catry et al., 2003). The emergence of AMR in veterinary medicine can therefore compromise animal health and welfare. In order to preserve and uphold this, sustainable changes in antimicrobial use (AMU) must be made (OIE, 2016). When considering the use of antimicrobials within veterinary medicine for use in animals, the main concern with their use lays within global agriculture, specifically the livestock industry. Here, the use of antimicrobials, as with human medicine, can encourage the emergence of resistant bacteria, which can either become disseminated into the environment or down the food chain, with the potential to eventually affect humans (Paphitou, 2013). The potential transfer of resistance between animals, the environment and humans and the link between them raises some important points of thought and widens the debate and approach with regards to AMR.

It is widely accepted that antimicrobials play an important role in the management of food producing animals due to the improvements in health, welfare and efficiency that they play. Antimicrobials in agriculture can be used to treat bacterial infections which present clinical symptoms that can compromise health and welfare. They can also be used in the prevention of infection via prophylaxis and in growth promotion (Gustafson and Bowen, 1997). AMU within the livestock industry has allowed for more productive and healthier animals which has therefore allowed in turn for higher quality produce for human consumption at a lower cost (Oliver et al., 2011). Administration of antimicrobial compounds differ across livestock production systems. In the dairy, beef and sheep industries, antimicrobials are generally administered on an individual case basis. However, in large scale intensive rearing systems, such as that for poultry and swine, antimicrobials may be delivered via water or feed for an entire group for example, as result of a few identified cases of illness (McEwan, 2006).

Livestock agriculture has been calculated to account for 73% of all AMU globally (Van Boeckel et al., 2019). It has been estimated that in 2017, 93,309 tonnes of antimicrobial active ingredient were used worldwide across swine, poultry and cattle agriculture, with an estimated increase of 11.5% to 104,079 tonnes of antimicrobial active ingredient by 2030 globally. Of the three livestock sectors reported, AMU increase in cattle was the lowest, accounting for only 22% of the overall increase by 2030 (Tiseo et al., 2020).

As previously described, the use of antimicrobials within agriculture places a selective pressure on bacteria, both pathogenic and commensal, within the host and poses a risk for the emergence and dissemination of resistant bacteria as well as their genes. These may be passed on down the food chain and may compromise human health. Additionally, resistances within livestock can compromise health and welfare, and limit the use of a range of antimicrobials to treat bacterial infection.

1.4 Political responses

1.4.1 International response

Greater recognition of the issue of AMR is being paid towards its impact on animal health, and that of the wider environment (Queenan et al., 2016). This therefore brings forth the concept of 'One Health', which has drawn increasing levels of attention in the past decade with regards to AMR. One health describes and develops upon the notion that there is a need for cooperation and communication between various professional disciplines at local, national and global levels to achieve improved health for all people, animals and the ecosystem in a global context. (Gibbs and Anderson, 2009). With the world witnessing increasing levels of international travel, trade and cooperation through expanding globalisation, this in turn provides an opportunity for an unprecedented movement of bacteria, along with those that display AMR properties. In essence, practices which encourage the emergence and spread of resistant microbes in one country can compromise the efficacy of antimicrobials on a worldwide scale (OIE, 2015). The OIE (World Organisation for Animal Health, formerly the Office International des Epizooties) calls for better "global, multi-sector" strategies to curb the emergence and spread of antimicrobial resistance. Inoue and Minghui (2017) suggest that it is necessary for governments around the world to begin to rapidly implement and adhere to a One Health approach, which would see close association between their respective health, agriculture and environmental offices. Emphasis on the importance and relevance of the One Health concept for the issue of AMR is widespread in the literature. For example, Robinson et al., (2016) claims that there is no other issue that embodies the fundamental ideas of the One Health approach than that of AMR, with Moran (2017) stating that it is the "quintessential planetary One Health challenge".

In response to the calls for greater political attention and collaboration on a global scale, there has been increasing momentum to bring about meaningful change in order to combat the scale of the problem. Despite the major political traction that has been generated within the last decade, the WHO began the discussion with

regards to the threat posed by AMR as early as the 1960s, a mere 20 years following the introduction of antimicrobial agents worldwide. The 'Resolution on Antimicrobial Resistance' was issued in 1998 and in 2001, the WHO, in following up from this, published their first so called global strategy (The Lancet Editorials, 2016). However, it has been claimed that the publication failed to achieve a meaningful response from the wider medical, scientific and political landscape due to a lack of emphasis on the economic implications (Queenan et al., 2016). The 1998 resolution and following 2001 strategy aimed to provide a series of frameworks for the member nations of the WHO to; prevent infectious outbreaks, slow the emergence and spread of resistant microorganisms and to also encourage the research and development of novel antimicrobial agents (World Health Organisation, 2001). Despite this perceived lack of a meaningful response, the WHO continues to lead the way in the recognition, monitoring and in confronting the scale of AMR.

The first report regarding AMR surveillance worldwide was published by the WHO in 2014 and in 2015 a new global strategy plan was published by the WHO and endorsed by the World Health Assembly (the decision making body of the WHO) (Barber et al., 2017). By this point, a great deal of attention had finally been given to the scale of the emergence and spread of resistance, and the collaboration which was being called for was achieved by the co-ordinated effort of the Food and Agriculture Organisation (FAO) and the OIE alongside the WHO in the publishing of their 2015 strategy. This collaboration began in 2010 in a so called 'tripartite alliance' to work together in promoting the One Health concept through their respective responsibilities, which is now readily focusing their efforts on AMR, among other One Health commitments (OIE, 2015). The OIE itself is also dedicated to formulating strategies with regards to antimicrobial resistance. At their 83rd General Session in 2015, the 180 nation members committed to the promotion of responsible use of antimicrobials in the world's animal population. The main themes that the OIE are committed to are; bettering perceptions on the use of antimicrobials, improving surveillance and research, encouraging better governance with respect to AMR and implementation of agreed international standards.

The scale of the impact has been epitomised by it reaching the highest levels of the global political landscape, namely the United Nations, when in 2016 the 71st General Assembly adopted a resolution aimed at combating the threat of antimicrobial resistance, as well as the G7 and G20 groups of nations (OIE, 2016). In addition to major strategic and monitoring reports being published by the high profile organisations such as the WHO, the FAO and the OIE, they have also worked jointly on the compiling of a list of antimicrobials according to their importance in the areas of human and veterinary medicine.

In 2005, the WHO presented their listings of antimicrobials and their importance in human medicine at a meeting in Canberra, Australia. Here, antimicrobials were grouped according to levels of importance; *critically important, highly important* and *important* (World Health Organisation, 2005). The list has been revised every other year since the original document was published in 2005.

Similarly, in May 2007 the OIE published a similar document at their 75th General Assembly (FAO/OIE/WHO, 2008), whereby antimicrobials were designated to be *critically important, highly important* or *important* in veterinary medicine, with the range of animal species being treated within veterinary medicine being accounted for.

1.4.2 European interventions

Doyle et al. (2013) state that the Swann Report of 1969, a UK parliamentary report, was the first extensive enquiry into the impact of the use of antimicrobial agents in the agricultural sector on the health of the human population. The report came to a number of conclusions, however it emphasised that the extensive use of antimicrobials in food producing animals was a hazard for human health (Swann et al, 1969). Following the recommendations within the report, the UK, Europe and Australia abolished the use of tetracyclines, penicillin and streptomycin, which were being used as growth promotors in agriculture. Other classes of antimicrobials began to be used in their place, such as virginiamycin and avilamycin (Barton, 2000). Europe was the first to lead the way in taking action on the use of antimicrobials being used

as performance and growth enhancers in animal feed. In 1986, Sweden became the first country to enact a ban on their use, and by 1988 had also introduced a prohibition on more generalised use of prophylactic AMU in agriculture. As a result, there was a reduction of antimicrobials sold for use in agriculture in Sweden from an average of 45 tonnes to an estimated 15 tonnes in 2009. Similar moves were made by the Danish government in the early 1990s (Cogliani et al., 2011). In 1995, the EU introduced a ban on the remaining four antimicrobial agents available for use in growth promotion; bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al., 2003). This left only avilamycin, flavomycin, monensin and salinomycin available for use as growth promotors in animal feed in the EU, but it was decided by the European Commission that these too should be phased out and a ban was introduced on 1st January 2006. Following this, direct application of antibiotics or use in medicated feed is only allowed under veterinary prescription (Anadon, 2006). It is largely accepted that the bans imposed in Europe made a significant contribution in the phasing out of growth promoting antimicrobials. Denmark has been widely regarded as having one of the best AMU surveillance procedures in Europe, and it has been shown that their use had fallen from 100 tonnes to zero by 2000 (Phillips, 2007). Many surveillance programmes and strategies have been introduced in the EU since, but more recently, a proposal to limit the availability of antimicrobials deemed to be critical in human medicine for use in veterinary medicine, came into effect in January (European Commission, 2018; More, 2020).

In the last 20-30 years, a number of governmental and medical research institute reports have been commissioned across the world. The common conclusion is that the key to mitigate the impact and development of AMR is to implement meaningful surveillance, greater commitment to research and infection control (to prevent AMU in the first place) and to encourage more prudent use of antimicrobials across human and livestock populations and the environment (Wise, 2002).

1.4.3 United Kingdom's response

As referenced to earlier, the Swann Report of 1969, commissioned and produced in the United Kingdom (UK), was the first scientific report on the risks that AMU in the livestock industry posed to public health (Begemann et al., 2018). Furthermore, the UK was seen to continue international leadership in the area through the publication of the O'Neill report in 2016, as alluded to previously. The report made seven recommendations, including promoting increased global awareness of antimicrobial resistance, reduction of unnecessary use of antimicrobials in agriculture and improved global surveillance of AMU and AMR (O'Neill, 2016). The report would influence policy not only in the United Kingdom, but also on a global level. In 2013, the UK government had outlined a five year AMR strategy, to be renewed in 2018, which brought together governmental departments concerning agriculture and the environment, health, associated public health agencies and their subsidiaries. However, the O'Neill report provided a so called 'catalytic impact', which received more attention than the original strategy itself (Blake et al., 2022).

In response, the Responsible Use of Medicines in Agriculture Alliance (RUMA), which was originally established in the UK in 1997 to "promote the highest standards of food safety, animal health and animal welfare", outlined targets and guidelines to help the livestock sector meet the recommendations of the O'Neill report. The RUMA Targets Task Force guidance helped the UK achieve a 52% decrease in AMU across the entire livestock sector between 2014 and 2020, with updated guidance and targets published for 2021-2024 (RUMA, 2022). A key element of the UK's surveillance system is the annual publication of the Veterinary Antimicrobial Resistance and Sales Surveillance (VARSS) report, compiled and published by the Veterinary Medicines Directorate (VMD). This report outlines key data relating to the sales of antimicrobials in livestock and AMR monitoring of bacteria (Bennani et al., 2021).

1.5 The dairy industry

The latest available global data shows that in 2019, global milk production stood at 883 million tonnes (81% produced by cattle). Of this output, Asia and Europe accounted for 42% and 26% in milk production respectively (FAO, 2021). The European Union dairy herd (ex-UK) stood at 20.5 million cows in 2020 while the UK milking herd was made up of 2.62 million cows (AHDB, 2022).

From their analysis of global trends, Tiseo et al., (2020) estimated AMU across food producing animals. Measures of AMU were presented as milligrams of active antimicrobial ingredient used per population correction unit (mg/PCU). This metric takes the size of the animal population into account using estimated weights of food producing animals (Veterinary Medicines Directorate, 2016). The authors estimated that in 2017, cattle (all dairy and beef) accounted for the smallest proportion of AMU of the food producing animals included in their work (swine, poultry and cattle). The authors found AMU for cattle to be at 42mg/PCU, compared with 193mg/PCU and 68mg/PCU for swine and poultry respectively. In a paper by Kuipers et al., (2016) the authors conducted a long term study into AMU from 2005 to 2012 in 94 dairy herds in the Netherlands, with data being obtained from veterinary sales data from each herd. Between 2005 and 2012, the use of third and fourth generation cephalosporins and fluoroquinolones fell from 18% of overall use to only 1%. This reduction however brought about an increase in use of penicillin and other β-lactam products as well as broad-spectrum products such as trimethoprim/sulfonamide combinations. Elsewhere, a study on Canadian dairy farms (Saini et al., 2012) found that β-lactams were the most widely used antimicrobial class across 84 dairy herds. It was noted that cephalosporins, tetracyclines, trimethoprim/sulfonamide based antimicrobials and lincosamides were frequently used (in descending order of use). Additionally, they concluded that the use of fluoroquinolones was relatively low.

Within the UK, it was found that the use of antimicrobials in the dairy industry was lower than that of the average across the livestock sector as a whole, with an average of 22.11 mg/PCU across a convenience sample of 358 dairy farms. It was determined

that the most commonly used antimicrobial products were β-lactams and aminoglycosides, which represented 42.8 % of ingredient mass (mg) and 20.9% respectively. Fluoroquinolone use along with third and fourth generation cephalosporin use accounted for an overall low proportion of use in terms of antimicrobial active ingredient (Hyde et al., 2017).

Within the dairy industry, the main usage of antimicrobials is in the treatment of mastitis, which can occur either during lactation or dry period (Swinkels et al., 2015) and is widely regarded as one of the most costly diseases within the dairy industry worldwide (Hand et al., 2012). Mastitis is the term used to describe an inflammatory disease of the mammary gland and is the result of entry of infectious agents and subsequent colonisation of the mammary gland, with the inflammatory response presenting itself as a result of a pathophysiological reaction to the presence of the infectious agents (Biggs, 2009). The aetiology of mastitis is almost always of bacterial origin (Royster and Wagner, 2015). In the United Kingdom, it has been determined that only five bacterial species account for 80% of mastitis diagnoses; *Escherichia coli, Streptococcus uberis, Streptococcus agalactiae, Streptococcus galactiae* and *Staphylococcus aureus* (Bradley, 2002). As a result of mastitis being of relatively high prevalence in dairy herds, this disease constitutes the main source of AMU. Many studies considering AMR in dairy often focus on mastitis causing pathogens, or opportunistic pathogens of the mammary gland.

1.5.1 Associations between antimicrobial use and resistance

Although there has been a great deal of work done to quantify AMU on dairy farms and changes in use over time, work focusing on the differences in AMU between farms and resistance patterns is limited. A study by Saini et al., (2013) which followed up on previous work quantifying AMU, found that there was an association between AMR in *E.coli* isolated in bovine mastitis cases and antimicrobials commonly used on Canadian dairy farms. In this work, the authors sampled individual cows on a quarter level basis according to three experimental groups; (i) those identified as having clinical mastitis, which were sampled prior to and following antimicrobial treatment,

(ii) non-clinical, healthy cows and (iii) pre-drying off and post calving cows. A total of 394 quarter level samples were obtained across 76 dairy farms which were believed to be geographically representative of Canadian farms. By sampling clinical mastitis cases, the authors were actively selecting for pathogens which are the primary cause of AMU on dairy farms, rather than a broad overarching surveillance programme. The authors demonstrated that the use of β-lactam antimicrobials were associated with an increased frequency of *E.coli* isolates showing intermediate resistance or higher to aminoglycoside based products, ampicillin and trimethoprim/sulfonamide combinations. In work elsewhere, Catry et al., (2016) carried out a two year antimicrobial susceptibility study evaluating isolates of *E.coli* sampled from the digestive tracts of 10 dairy, 10 beef and 5 veal herds in Belgium, with study groups ranging from 144 to 594 individuals. Milk sampling would have been inappropriate due to the inclusion of beef and veal study groups. A notable finding was that higher use of antimicrobials had a strong influence over the frequency of AMR in *E. coli*. Higher levels of AMU were found amongst veal herds compared to dairy and beef, but the authors concluded that this increased the incidence of AMR in commensal and pathogenic bacteria.

Research of interest has been carried out in South Korea investigating changes in antimicrobial susceptibilities of Gram-negative bacteria, including *E.coli*, isolated from cases of bovine mastitis over an extended period. Nam et al., (2009) found no significant change in the incidence of AMR in Gram-negative bacteria between 2003 and 2008. In total, 841 Gram-negative isolates were retrieved, of which 161 were E. coli. The authors noted that the lack of any significant change in antimicrobial susceptibilities across such a long monitoring period was unexpected. A similar longitudinal style study from South Korea investigating the antimicrobial susceptibilities of *E. coli* isolates (n= 374) between 2012 and 2015, also found no significant changes in the incidence of AMR during this time. However, it was noted that the rates of resistance were similar when compared with the aforementioned 2009 study (Tark et al., 2017). These studies had no data on AMU for the cows or herds from which samples had been taken. Therefore, the effect that differences in AMU may have on the results relating to the incidence of resistance in these studies

is unknown. Given the role that AMU has been found to play in the emergence of AMR, consideration of both these areas is important in any surveillance programme, with additional research being required to determine the relationships between these at the farm level.

1.6 Farm management

In addition to AMU, the influence of farm management system (e.g. conventional vs organic) on AMR has been proposed and it is suggested that management is the most important factor related to resistance after AMU (Murphy et al., 2018). These authors noted that although conventional and organic systems were identified as a point of importance, these systems may represent a range of many practices relating to farm management, such as housing, biosecurity and farm density. Such factors may play an important role in the emergence of AMR, either through direct associations or indirectly, by encouraging increased AMU.

In one study, it was found that when compared with conventional dairy farms (n= 30), there was a significantly lower level of resistance in isolates of *E.coli* cultured from faecal samples from organic dairy herds (n=30) (Sato et al., 2005). In a more recent study from Germany comprising a larger number of farms (303 organic and 372 conventional systems) investigated the incidence of methicillin-resistant *Staphylococcus aureus*. It was found to be more frequently isolated from the bulk tank milk of conventional dairy farms than from organic systems (Tenhagen et al., 2018). Although these findings may be simply related to differences in AMU, these farming systems represent differences which farm management can play when considering AMR. This therefore highlights the potential for further detailed simultaneous investigation of both AMU and general farm management practices to fully understand the dynamics of AMR on dairy farms.

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1.7 Antimicrobial resistance monitoring and surveillance

The potential risk for the emergence and dissemination of AMR between bacteria and amongst hosts warrants judicious monitoring of bacterial susceptibilities. The implementation of surveillance procedures allows for informed decision making to occur, including directing new policy approaches as well as informing the direction of future surveillance. Laboratory generated data allows for determination of susceptibility or resistance of bacteria to antimicrobials, allowing for new or emerging trends to be monitored longitudinally and/or in areas of geographic interest (Johnson, 2015).

1.7.1 Sampling methods

When studying or monitoring AMR in the dairy environment, there are a number of options available in terms of sampling, such as individual quarter milk samples (Thomas et al., 2015) or faecal/bedding samples taken from housing facilities or the wider farm environment (Alzayn et al., 2020). However, it has been suggested that bacteria isolated from milk samples taken from the farm's bulk tank may be of value, which represents a convenient sampling method and has been assumed to be is representative of the herd population and its environment (Berge et al., 2007). Many studies into AMR on dairy farms report using bacteria isolated from bulk tank milk, across a range of bacterial species (Del Collo et al., 2017; Kreausukon et al., 2012).

1.7.2 Sentinel bacteria

One way to carry out surveillance on antimicrobial resistance and monitor trends or developments is by using sentinel bacterial species. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) use *Enterococcus* spp. and *Escherichia coli* as proxy sentinel bacterial species in the monitoring of AMR. The former allow for monitoring of resistance in Gram-positive bacteria, with *E. coli* allowing for monitoring of resistance in Gram-negative bacteria.

These bacterial species hold value for a number of reasons. Firstly, they are ubiquitous in the environment of livestock. Secondly, they form part of the natural makeup of the host microbiota in the gastrointestinal tract in both animals and humans. Finally, they can rapidly acquire and develop resistances to a range of antimicrobial agents and therefore have the ability to disseminate them across the bacterial population to members of the same species but also to others as a result of bacterial gene transfer (Borck Høg et al., 2016). Therefore, the use of these indicator bacterial species play an important part of studies in AMR patterns. It is for these reasons that *Enterococcus* spp. and *E. coli* are the focus of investigations in AMR in this thesis.

1.7.3 *Enterococcus* spp.

The *Enterococcus* are a genus of bacteria of significant interest in both the fields of medicine and science. *Enterococcus* are categorised as Gram-positive, oxidase and catalase negative, non-spore forming bacteria and are naturally present in a range of environments including water, soil, and form part of the natural microflora of the gastrointestinal tract of both humans and animals. Crucially, *Enterococcus* have been found to be part of the normal microbiota of unpasteurised milk (Čanigová et al., 2016). Over the past number of decades, *Enterococcus* spp. have become a major cause of infections associated with medical treatment with the UK reporting a 60% increase in bloodstream infection of *Enterococcus* origin between 2002 and 2007 (Heimer et al., 2014).

Enterococcus spp. hold particular interest with regards to AMR due to the fact that they are inherently resistant to a range of antimicrobial agents, such as cephalosporins, penicillin derivatives, lincosamides and aminoglycosides. Due to the selective pressure of AMU, resistance among Enterococci have emerged to a number of other agents, such as streptogramins and tetracycline (Heimer et al., 2014). Jackson et al., (2011) found that amongst *E. faecalis,* resistance to lincomycin was highest at 97.8% of isolates, with the next highest resistance being to tetracycline at 12.2%. *E. faecium* showed high levels of resistance against flavomycin (88.2%) and

lincomycin (81.2%) but a lower level of resistance to tetracycline (8.2%). When *E. durans* was tested, 100% resistance (of 22 *E. durans* isolates) were resistant to lincomycin and 36.4% of isolates were resistant to flavomycin and tetracycline. In a separate study (Nam et al., 2009), it was found that the most common resistances among *Enterococcus* spp. isolates were to tetracycline (69.5%), penicillin (64.7%) and erythromycin (57.1%). These results suggest the need for further surveillance of AMR patterns to better monitor any changes or the emergence of new resistances.

1.7.4 *Escherichia coli*

Escherichia coli are Gram-negative, rod shaped bacilli belonging to the *Escherichia* genus and are a part of a group of bacterial species known as coliforms. With respect to research in the area of dairy science, *E. coli* represent a significant proportion of coliform species associated with mastitis (Suojala et al., 2013), acting as an opportunistic pathogen of the udder. *E. coli* play an important role as commensal bacteria in the gastrointestinal tracts of humans and animals, meaning that *E.coli* has a considerable presence in the faecal content of dairy cows. It is therefore widespread in the dairy environment (Keane, 2016).

Much work has been carried out on the antimicrobial susceptibility of *E. coli* implicated in bovine mastitis, with a large degree of variability in results between studies. A study undertaken by "VetPath", a pan-European association that provides monitoring of AMR in livestock, reported that *E. coli* isolates were most commonly resistant to the antimicrobial agents cefapirin (11.1% of isolates) and 14.3% of isolates were resistant to tetracycline. Other antimicrobial agents were also tested with isolates showing either zero or minimal levels of resistance towards them (Thomas et al., 2015). A study by (Suojala et al., 2011) in Finland reported 27.8% of 144 *E. coli* isolates showed resistance to at least one antimicrobial, with multidrug resistance being seen in 20.1% of isolates. In this study isolates were most commonly resistant to ampicillin, streptomycin, tetracycline and sulfametoxazole (18.6%, 16.4%, 15.7% and 13.6% respectively). A 2007 study from the USA (Srinivasan et al., 2007) found extremely high levels of resistance amongst *E. coli* to antimicrobials

commonly used in veterinary medicine. It was found that all isolates were deemed multi-drug resistant with 98.4% of isolates being resistant to ampicillin, 40.3% resistance for streptomycin and 24.8% for tetracycline. Therefore, conclusions were drawn that *E. coli* may represent an important reservoir of AMR genes and allow for propagation of resistance amongst commensal and pathogenic bacterial populations. This in itself highlights the importance of *E. coli* as a sentinel bacteria in AMR monitoring programmes.

1.8 Methods of measuring antimicrobial resistance

Determination of AMR can be achieved through a number of methods, but can be grouped according to phenotypic or genotypic assessment of resistance (Anjum, 2015). The most frequently employed approach is via phenotypic identification, where bacteria are grown in the presence of antimicrobial agents. Molecular based methods are growing in popularity, for example, through the use of genetic sequencing (Feldgarden et al., 2019). This allows for the identification of AMR related genes which could be passed on throughout the bacterial population via horizontal gene transfer (von Wintersdorff et al., 2016). Classification of these genes and a high sample throughput are key benefits of a molecular approach (Kaprou et al., 2021).

In contrast, phenotypic methods in the determination of AMR have been well established and standardised (Qi et al., 2006) via antimicrobial susceptibility testing (AST). A number of individual methods employing this approach exist, such as agar disc diffusion, agar dilution and broth macrodiluton or microdilution (Jenkins and Schuetz, 2012). AST regimens which determine the minimum inhibitory concentration (MIC) via dilution methods are widely recognised as the 'gold standard' (Schumacher et al., 2018). The MIC is a measure of the lowest concentration of an antimicrobial needed to inhibit growth of microbes, such as bacteria (Ericsson and Sherris, 1971). Broth microdilution (BMD) is one of the most widely used methods in the determination MICs of bacterial isolates (Jorgensen and Ferraro, 2009) and utilises 2 fold serial dilutions of antimicrobials usually expressed in concentrations of µg/ml. Pure cultures of bacterial isolates are incubated in the presence of the

antimicrobial agent and the MIC subsequently determined. The MIC measure however suffers from inaccuracies due to biological and assay variation, pharmacokinetic relationships, and also error around the 2 fold serial dilution (Mouton et al., 2018). Therefore, there is an accepted MIC error of ± one microdilution when considering the MIC of a given isolate (International Organization for Standardization, 2007).

Despite this and due to its widely recognised reputation as a gold standard of AMR determination, the MIC was used as a measure of resistance of bacterial isolates investigated throughout this PhD project.

1.9 Aims and objectives

In the context provided by this literature review, the overall aim of this research project was to further our understanding of the dynamics concerning AMR with a particular focus towards dairy farms. This was to be addressed through three main objectives.

The first of these objectives was to investigate the associations between AMU and AMR via implementation of a longitudinal study utilising historic data. Investigation of this aim is presented in Chapter 2 and was further explored in Chapter 6.

The second objective was to investigate potential non-AMU related factors associated with AMR in the context of dairy herds through consideration of farm management practices. This was to be addressed via a cross sectional study as presented in Chapter 3.

The third and final objective was to explore the viability of a novel laboratory approach utilising BMD which could be employed as part of a routine AMR monitoring programme through sampling of bulk tank milk. This work is outlined and presented across Chapters 4 and 5.

Chapter 2; A longitudinal study of antimicrobial use and resistance

2.1 Introduction

In Chapter 1, the relationships between AMU and AMR were considered, whereby the selective pressures placed by antimicrobials on bacterial population leads to emergence of resistance. Further knowledge of the causal relationships which exist between AMU and AMR with specific reference to dairy herds would aid future decision making, both at the veterinary clinician and policy levels. Studies referred to in Chapter 1 present long term consideration of AMU and AMR both separately and together to establish trends and relationships. In this chapter, a longitudinal study of the associations between AMU and AMR is presented. AMU was inferred from veterinary sales records, and AMR measured in terms of the MICs of sentinel bacterial species; *Enterococcus* spp. and *E.coli*.

A study population of sixteen dairy farms located on an isolated geographic location were recruited for study. Existing contacts were already present between the PhD industrial partner (Quality Milk Management Services Ltd.) and the farmer owned dairy cooperative. Interest in research had already been expressed on both sides, so therefore represented an opportunity for convenient farm recruitment. Secondly, all dairy farms on the island supplied the farmer owned dairy cooperative with their milk, excluding one independent dairy enterprise. This therefore represented a unique opportunity for data capture of almost an entire population. Finally, restrictions on importation of cattle to the island meant that any potential patterns were arising solely within the study population. With the links between QMMS and the dairy cooperative having existed for some time, an archive of frozen bulk tank milk samples from individual farms had been stored at QMMS. This represented a pool of historic samples with the potential for a longitudinal study over a number of years to be carried out.

2.2 Materials and methods

2.2.2 Farm recruitment

Initial contacts between researcher (DM) and the dairy cooperative were facilitated by QMMS. Prior to this, farmers had been informed of a potential study, with their recruitment having been encouraged by the cooperative. Formal recruitment was organised by email and/or phone. Prior to the commencement of the study, there were a total of 19 dairy farms on the island. Two herds were undergoing a merger to form one unit and one farm was independent of the dairy cooperative. Therefore 17 herds comprised the target population. Of these, 16 agreed to participate in the study (94% response rate). Farmers were asked to sign consent forms to agree their participation in the project and that all data shared during the course of the study would be confidential, anonymous and would be handled according to General Data Protection Regulation (GDPR) regulations.

2.2.3. Farm Visits

Farm visits, during which a questionnaire was carried out with farmers, will be further discussed in Chapter 3. In brief, an initial scoping visit to the island took place in January 2019. Visits were made to the dairy cooperative and the two veterinary practices which provided services to the island's dairy farmers. Following visits to all participating farms, veterinary practices were approached again for the purposes of antimicrobial sales data collection.

2.2.4 Antimicrobial sales data

2.2.4.1 Data retrieval, sorting and collation

Antimicrobial sales data were obtained from veterinary practice records as a proxy for on farm AMU. Data were collected from the two veterinary practices, representing the farmer client base. Across the sixteen farms recruited to the study,

there was an equal distribution of farmers across the two practices. The veterinary practices were initially contacted to inform them of the study. An initial in person visit was made to discuss the data needed and logistics for obtaining this. Veterinary records were held electronically on two different software programmes; ezyvetpro (ezofficesystems, Hampshire, UK) and teleosvet (Teleos Systems Limited, Birmingham, UK). For all farm accounts (eight at each veterinary practice) and on both systems, records were initially filtered by date to obtain records from April 2013 to April 2019, and then by treatment type (antimicrobial). These records encapsulated AMU from at least one year before bulk tank samples began being archived at the laboratory (August 2014) until the point of data retrieval. Records were exported as comma separated values (CSV) files and saved electronically. The data output from this system included information relating to; date of sale/product prescribed, client details, treatment item (including quantity and type), prescribing veterinarian, product sale costs and a number of personal details related to the client. Data outputs from the second veterinary practice included information relating only to the client's address, data of sale/product prescribed and details relating to the product (name, quantity and directions for use).

In addition to antimicrobial purchases and prescriptions from their veterinary practice, six farmers indicated that they used an online veterinary medicine distributor. To obtain these data, the distributor was approached. Once farmer's consent was granted and shared with the distributor, sales data were forwarded via email. These data included information relating to date of product purchase/dispense, quantity and a number of points of information relating to client's personal information.

Data were handled in an Excel spreadsheet (Microsoft Excel, Microsoft Corporation, 2016). From records obtained, data were cleaned by removing unnecessary information such as clinical indications and client details to leave that only relating to date, antimicrobial item and quantity used/dispensed. Raw data indicated whether a whole bottle or a specified volume of antimicrobial had been dispensed.

For each farm's records, columns were added to recode the information present. Recoding included; a simplified product name/identifier, simplified quantity of

product (in ml) and information relating to the active ingredient(s) of each antimicrobial product. To define active ingredient information, a database produced by the Veterinary Medicines Directorate (Veterinary Medicines Directorate, 2019) was accessed , with the database being filtered to show products across four antimicrobial categories. The information for each antimicrobial product provided by this database allowed for determination of the active substance(s) and its pharmaceutical form (i.e. solution for injection, oral solution or intramammary suspension). This was used to define which active substance(s) constituted the product, and what antimicrobial class it belonged to e.g. ceftiofur (a cephalosporin) was assigned as a third generation cephalosporin. Where a product contained two or more active ingredients, each individual component of the product was defined in the same manner. Also provided in the Veterinary Medicines Directorate database were links to the product data sheets, which indicated the quantity of active ingredient contained per 1ml of solution, or in the case of intramammary suspension, per syringe, in milligrams. These data were used to calculate the total amount of antimicrobial active ingredient that was dispensed/purchased on a given instance. For each record entry from the sales data, the total amount of antimicrobial was calculated, in grams of active substance. Additionally, a calculation was made to determine antimicrobial amounts per cow, in which the total antimicrobial amount for each record point was divided by the number of cows in that farm's herd. All sales records for the sixteen farms were collated and handled accordingly.

2.2.4.2 Descriptive and graphical analysis

Following data handling, initial descriptive analysis was carried out to identify antimicrobial classes with the highest frequency of use between April 2013 and April 2019 across all farms. Further descriptive analysis was carried out to assess the changes in AMU patterns within farms. For each farm, a measure of AMU was calculated using the Population Correction Unit (Veterinary Medicines Directorate, 2016) in terms of mg/PCU, according to the formula;

$$x \text{ mg} / y \text{ kg} = \text{mg/PCU}$$

where x is equal to antimicrobial in milligrams and y is equal to mass in kilograms of all dairy cows on the farm, using a standardised figure of 425kg for a dairy cow.

A graphing procedure using the *ggplot2* package (Wickham, 2016) in R (RStudio Team, 2020) allowed for a visual representation of AMU patterns for each farm. From the datasets prepared for each farm, data was transformed to include that relating to date and total antimicrobial (in grams) for each antimicrobial class only, and subsequently plotted quarterly (according to date data). Additionally, graphs were also produced for data relating to AMU on a per cow basis.

2.2.5 Generation of antimicrobial susceptibility data

2.2.5.1 Recovery of bacterial isolates

Isolates of *Enterococcus* spp. and *E. coli* were recovered from frozen bulk tank milk samples stored by the laboratory since August 2014. Bulk tank samples had been archived annually until August 2018, after which samples were archived on a seasonal basis (November 2018; February 2019, August 2019 and November 2019). Both bacterial species were recovered from milk fat, which had been identified as an enriched culture medium. Bacterial cultures were subsequently grown on selective agars. Once defrosted, the milk was pre-incubated for two hours at 37°C. Following incubation, samples were inverted to allow the milk to mix. For each farm, 10-12ml of milk was transferred into three sterile falcon tubes which were then centrifuged for two minutes at 4000rpm. Once spun, each falcon tube provided enough milk fat to be spread across selective two of each selective agar plate. Slanetz and Bartley (SB) agar was used in the selection of *Enterococcus* spp. and Tryptone Bile X-Glucuronide (TBX) agar in the selection of *E. coli*. Sterile cotton swabs were used to take half the milk fat from each falcon tube, which was then distributed onto six SB and TBX agar plates. Approximately 300µl of milk supernatant was added to each plate to allow for better spreading of fat. The contents of each plate were mixed using a spreader to

create a smooth consistency and then spread evenly across the whole plate. Once dry, plates were incubated for 48-72 hours at 44°C and checked after 48 hours for growth. At 48 hours, any plates that lacked growth were discarded. Plates that did not have significant growth were left until 72 hours had elapsed and rechecked. In addition to TBX agar, liquid growth media were also used in the recovery of *E. coli*. For each farm's bulk tank sample, 1ml of milk was incubated in 5ml of Enterobacteriacae enrichment (EE) broth for 18-24 hours at 44°C. For each farm, aliquots of 200µl and 500µl of bacterial suspension were plated onto TBX and VRBG agar.

Following the incubation period, plates that featured significant growth of contaminants (i.e. where two or more contaminant colonies were identified morphologically) were discarded. Eight to ten colonies of *Enterococcus* spp. and *E. coli* per farm were selected from the six SB or TBX plates. Colonies were selected by visual assessment of morphology. When there was growth across all six plates, colonies were selected from all plates to obtain a variety of strains. If any TBX plates (plated from milk fat) did not grow sufficient colonies, TBX and VRBG plates, which had been plated from EE broth, were checked for colony growth.

Species identification was subsequently confirmed through Matrix-Assisted Laser Desorption Ionization/Time of Flight Mass Spectrometry (MALDI-TOF MS) using Biotyper 3.1 (Bruker Daltonics, Coventry, UK). Bruker sheets, which represented the layout of the MBT Biotarget 96 plates (comprising 96 sample positions) used by the MALDI for bacterial identification, were labelled according to the plates and colonies previously identified. For each MALDI run, the first spot on the plate was reserved for an *E. coli* control, and the second was left blank as negative control. Samples were placed on a target spot using a cocktail stick. Colonies were touched lightly, then transferred to the spot and spread down. This was repeated for all bacterial colonies to be identified. A 1µl aliquot of 70% formic acid was pipetted on each spot and left to dry. Once dry, the plate was matrixed by pipetting 1µl of Bruker Matrix HCCA (HCCA; α-Cyano-4-hydroxycinnamic acid) on top of each spot and left to dry. While drying, the MALDI Biotyper software was prepared for bacterial identification. Details of the Bruker sheet labelled previously were transferred to a pre-prepared Excel

spreadsheet, copied into the software and a new classification and project file created. Once ready, the target plate was placed into the Microflex™ mass spectrometer and loaded into the machine. Once the vacuum was ready, the procedure was run. Once completed, results were displayed in the software and copied to an Excel spreadsheet, saved to a USB and printed for future reference.

Colonies that were identified as being either *Enterococcus faecalis, Enterococcus faecium, Enterococcus durans* or *E. coli* were accepted for pure plating. At least six colonies were required for antimicrobial susceptibility testing (AST). If six colonies could not be obtained, milk samples were handled according to the method previously stated, using milk remaining in the 500ml sample which had been stored frozen. If none of the attempts to recover at least six *Enterococcus spp.* or *E.coli* colonies were successful, then a further milk sample was requested for processing at the next available date, which was usually 2 weeks after the initial delivery. This was, however, only possible for bulk tank samples arriving on and after August 2018. Following recovery, isolates selected for testing were pure plated on Columbia (5% sheep blood) agar and labelled according to sample ID, species and farm. Pure plates were placed in cold storage (approximately 4°C) with isolates being subsequently suspended on glycerol beads and stored at -80°C using the Protect Microorganism Preservation System (Technical Service Consultants Ltd, Heywood, UK) until ready for antimicrobial susceptibility testing. Table 2.1 outlines for which bulk tank milk samples each bacterial species were isolated and cultured for. Recovery of *E. coli* from archived frozen samples was unsuccessful.

Table 2.1~~2.1~~. An outline of bacterial species recovered from historic samples according to the period bulk tank milk samples were received by the laboratory.

Sample delivery date	E.coli	*Enterococcus spp.* (E. *faecalis,* E. *faecium* & E. *durans)*
August 2014	x	✓
August 2015	x	✓
August 2016	x	✓
August 2017	x	✓
August 2018	✓	✓
November 2018	✓	✓
February 2019	✓	✓
August 2019	✓	✓
November 2019	✓	x

2.2.5.2 Antimicrobial susceptibility testing

All AST was carried out between October and December 2019. Bacterial isolates to be tested were pure plated onto fresh Columbia (5% sheep blood) agar from those which had been in cold storage following bacterial recovery. Where contamination had occurred or if the isolates could not be revived, isolates suspended on glycerol beads frozen at -80°C were used for the pure plating process. All plates were labelled accordingly. When plating from cold stored isolates, a 10µl loop was used to transfer a portion of the colony onto the new plate, and was spread over a quarter of it, allowing for four samples per plate. Where bacteria were plated from glycerol beads, a single bead was selected using a bead pick and spread across a quarter section of the plate. Once plates were prepared, they were incubated for 18-24 hours at 37°C.

Following incubation, isolates were ready for AST, following the steps outlined by Thermofisher's Sensititre procedure (Thermo Scientific; Massachusetts, USA), in which results are generated as minimum inhibitory concentrations (MICs). For each sample, sterile cotton swabs were used to transfer bacteria from the pure plates into a vial of demineralised water. A standard bacterial suspension in demineralised water equalling a 0.5 MacFarland turbidity standard, equalling an optical density of

bacterial suspension of 1.5 x 10^8 colony forming units (CFU/ml) was determined according to a Sensititre Nephelometer (Thermo Scientific, Massachusetts, USA). For *Enterococcus spp.* isolates, a 30µl aliquot of bacterial suspension was added to a vial of 11.5ml of Mueller-Hinton broth. For *E. coli* isolates, a 10µl aliquot was added to the Mueller-Hinton broth. The vial was inverted two to three times to ensure mixing, after which the lid was replaced with a Sensititre single use dosing head. The vial was then placed in a Sensititre Automated Inoculation Delivery System (Thermo Scientific, Massachusetts, USA). A 96 well microdilution plate (Sensititre COMPGN1F) was subsequently inoculated with 50µl of bacterial suspension. The antimicrobials and the range of their respective concentrations across the COMPGN1F plate are provided in Table 2.2**.**

Table 2.2~~2.2~~. An outline of antimicrobials included on the COMPGN1F microdilution plate and their respective range of concentrations.

Antimicrobial	Concentration Range (µg/ml)
Ampicillin	0.25 - 8
Amoxicillin/Clavulanic Acid	0.25/0.12 – 8/4
Amikacin	4 – 32
Cefazolin	1 – 32
Cefovecin	0.25 – 8
Cefpodoxime	1 – 8
Ceftazidime	4 – 16
Cephalexin	0.5 – 16
Chloramphenicol	2 – 32
Doxycycline	0.25 – 8
Enrofloxacin	0.12 – 4
Gentamicin	0.25 – 8
Imipenem	1 – 8
Marbofloxacin	0.12 – 4
Orbifloxacin	1 – 8
Piperacillin/Tazobactam	8/4 – 64/4
Pradofloxacin	0.25 – 2
Tetracycline	4 – 16
Trimethoprim/Sulfamethoxazole	0.5/9.5 – 4/76

Following inoculation of the plate, an aliquot of inoculant was taken from one of the positive control wells with a 1µl loop and plated onto Columbia (5% sheep blood) agar as a test for inoculant purity, which was incubated for 18-24 hours at 37°C. An adhesive cover was placed over the plate and was incubated at 35°C for 18-20 hours. Following incubation, the plate was read using the Sensititre Vizion and SWIN™

software (Thermo Scientific, Massachusetts, USA). MIC results were then exported as text files and converted to Excel files. Individual result files and were collated in a master Excel file for later data handling and analysis.

Further AST with Micronaut-S Mastitis 3 microdilution plates (Merlin, Bornheim-Hersel, Germany) was carried out to provide a broader range of antimicrobials tested. Preparation of pure cultures was the same as previously described, as well as preparation of bacterial suspension in demineralised water. The range of antimicrobials and respective concentrations within the Mastitis 3 plate are provided in Table 2.3**.** For *Enterococcus spp.* isolates, an aliquot of 100µl of bacterial suspension was added to 11.5ml of Mueller-Hinton broth. For *E. coli*, an aliquot of 50µl was added to Mueller-Hinton broth. Vial lids were replaced with single use Sensititre dosing heads and placed in the Sensititre Automated Inoculation Delivery System. The 96 well microdilution plate was split in two, allowing for two samples to be tested per plate. The first 48 wells of the plate were inoculated with 100µl of bacterial suspension, with the remaining 48 wells being inoculated with 100µl of the following sample to be tested. Following inoculation, the plates were handled and incubated in the same way as the COMPGN1F plates. After incubation, the plates were placed in the Sensititre Vizion, with MICs being read manually, due to lack of compatibility between Micronaut-S Mastitis 3 plates and the SWIN™ reading software. Results were entered into an Excel spreadsheet for later analysis.

Table 2.3~~2.3~~. An outline of antimicrobials included on the Micronaut-S Mastitis 3 microdilution plate and their respective range of concentrations.

Antimicrobial	Concentration Range (µg/ml)
Ampicillin	4 - 16
Amoxicillin/Clavulanic Acid	4/2 – 32/16
Cefazolin	4 – 32
Cefoperazon	2 – 16
Cefquinome	1 – 8
Erythromycin	0.125 – 4
Kanamycin/Cephalexin	4/0.4 – 32/3.2
Marbofloxacin	0.25 – 2
Oxacillin	1 – 4
Penicillin G	0.125 – 8
Pirlimycin	1 – 4

2.2.5.3 Data Cleaning & Visual Analysis

Following completion of AST, all data outputs were collated, sorted and cleaned. Raw output data from the SWIN™ software consisted of sample IDs, organism and the MICs of all antimicrobials tested as well as interpretations of whether isolates were deemed susceptible, intermediate or resistant according to clinical breakpoints established by the Clinical and Laboratory Standards Institute (CLSI). Where CLSI breakpoints were not available, interpretative criteria provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used. This was merged with a secondary Excel file containing information relating to sample IDs, farm name and a corresponding ID number, organism and sample delivery date. Sample ID was used as a common heading to allow for merging the datasets using the *merge* function included in the base package of R software. Raw outputs pertaining to the Micronaut-S Mastitis 3 microdilution plates had been collated in a separate master Excel file. This was merged with the previously merged file using the common heading of sample ID, resulting in a final data file containing necessary information relating to sample and farm identifiers, sample delivery dates, organism and MIC data for both sets of antimicrobials tested.

To identify potential patterns in MIC data across the study period (August 2014 – November 2019), data were visualised graphically. Due to the breadth of data obtained across six samples for *Enterococcus* spp. and *E. coli* for each of sixteen farms, across a six year monitoring period, an overview of trends was generated using a mean MIC. To overcome the differing MIC ranges of each antimicrobial tested, each MIC data point was first rescaled from its tested concentration range to a standardised scale. This meant the MIC values for all antimicrobials were rescaled to cover the same range. This approach was based on the number of microdilutions of each antimicrobial and calculated according to the following equation;

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

Following rescaling, the mean MIC of the six isolates from each sampling period for both bacterial species was calculated, providing a single MIC value for each antimicrobial (to aid analysis of trends over time).

Following transformation of data, a graphical procedure of the mean MIC values was carried out using the *ggplot2* package in R (Wickham, 2016). For each farm, two graphs were produced; one for each of the two bacterial species. Graphs were examined visually to identify potential patterns of interest.

2.2.6 Analysis of associations

The associations between AMU and bacterial MICs were investigated. The spread and variability of MIC data for all sampling periods were considered in terms of descriptive analysis across all antimicrobials tested. A number of antimicrobials that varied little in terms of MIC across the entire period of 2014-2019 were identified, with these being deemed to be of little value in identifying possible associations with AMU. Antimicrobials considered in statistical analysis are shown in Table 2.4 and Table 2.5.

Table 2.4~~2.4~~. Antimicrobials included on the Sensititre COMPGN1F antimicrobial susceptibility plate which were selected for analysis, with their respective range of tested concentrations.

Antimicrobial	Concentration Range (µg/ml)
Ampicillin [a,b]	0.25 - 8
Amoxicillin/Clavulanic Acid [a,b]	0.25/0.12 – 8/4
Doxycycline [a,b]	0.25 – 8
Enrofloxacin [a,b]	0.12 – 4
Gentamicin [a,b]	0.25 – 8
Marbofloxacin [a]	0.12 – 4

[a] Antimicrobials tested against *Enterococcus* spp.
[b] Antimicrobials tested against *E. coli*

Table 2.5~~2.5~~**.** Antimicrobials included on the Micronaut-S Mastitis 3 antimicrobial susceptibility plate which were selected for analysis, with their respective range of tested concentrations.

Antimicrobial	Concentration Range (µg/ml)
Cefquinome [a,b]	1 – 8
Erythromycin [a]	0.125 – 4
Penicillin G [a]	0.125 – 8

[a] Antimicrobials tested against *Enterococcus* spp.
[b] Antimicrobials tested against *E. coli*

Prior to conducting statistical analysis, initial visual analysis was performed by constructing boxplots to explore the relationships between AMU and MICs. For MIC data, the mean MIC for each antimicrobial for each sampling period (as previously described) was used. For AMU, all data relating to each antimicrobial class, rather than individual antimicrobials was used to investigate associations with mean MICs of antimicrobials as outlined in Tables 2.4 and 2.5. The antimicrobial classes which were represented by selected antimicrobials were; aminoglycoside, β-lactam, cephalosporin, fluoroquinolone, macrolide (*Enterococcus spp.* only) and tetracycline. For example, when considering ampicillin MICs, AMU of all β-lactam antimicrobials was used in analysis. A binary approach of whether or not the relevant antimicrobial class was used within the six months prior to the sample delivery date was utilised. Here, a "1" indicated use, while "0" indicated zero use. A six month cut off was used as an assumption that potential changes in MIC may be most likely to occur within this period of time.

Subsequent statistical analysis of the associations between MICs and AMU was conducted using linear regression according to the following equation;

$$Yi = \beta 0 + \beta 1 Xi + \text{eij}$$

where Yi represented the dependent variable (mean MIC), $\beta 0$ represented the intercept, $\beta 1$ denoted the coefficient, Xi represented the independent variable (AMU) and eij represented the unknown error of the model.

Data used for linear regression was in the same format as that used in the construction of boxplots. Linear regression models were constructed for all antimicrobial classes representing antimicrobials selected for analysis against *Enterococcus* spp. Boxplots highlighted extremely low variation between MICs and use/non-use of antimicrobial classes in *E. coli* isolates. Therefore, construction of linear regression models was deemed to be of little value. The cut-off for statistical significance of all linear regression models was defined as P<0.05.

A second level of modelling investigating associations between MICs and AMU for *Enterococcus* spp. was performed via a mixed effects approach. Raw AMU data from the six months prior to sample delivery (rather than a binary approach as previously described) was used to investigate differences between farms, where farm name was assigned as a random effect. The mixed effects linear regression models were built according to the equation;

$$Yi = \beta 0 + \beta 1 Xi + \mathrm{Uj} + \mathrm{eij}$$

where Yi represented the dependent variable (mean MIC), $\beta 0$ represented the intercept, $\beta 1$ denoted the coefficient, Xi represented the independent variable (AMU), Uj denoted the separate effects of each mixed effect unit (farm unit) and eij represented the unknown error of the model. Uj and eij were assumed to be normally distributed with mean = 0 and variance Σ_u or Σ_e.

Statistical significance was set using the *t*-value, in which a value of >1.96 or <-1.96 were deemed significant (equivalent to P<0.05).

Two further instances of statistical analysis were carried out using linear regression and mixed effects linear regression. These procedures were the same in principle as the initial analysis using total AMU as the independent variable and mean MIC as the dependent variable, but used two different metrics to measure AMU, rather than total AMU. The first of these subsequent analyses used the metric defined daily dose (DDD) and considered only use of intramammary tubes for lactating cows. The second used defined course dose (DCD) and considered use of intramammary tubes for

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lactating cows and the use of dry cow tubes. As with the initial statistical analysis of total AMU, use of these intramammary tubes in the six months prior to bulk tank sample delivery was considered in analysis.

2.3 Results

2.3.1 Antimicrobial use descriptive results

Analysis of veterinary sales records highlighted a high degree of variation between farms in terms of the quantities of antimicrobials used and the number of different antimicrobial classes used on each farm. These classes were; aminocoumarin, aminoglycoside, β-lactam, cephalosporin, fluoroquinolone, lincosamide, macrolide, trimethoprim/sulfonamide and tetracycline. Four of the sixteen farms used all nine classes at least once during the six year period. The least variation in AMU was on one organic farm, where a maximum of five classes were used at least once across the period. The most commonly used antimicrobial classes across all farms were aminoglycosides, β-lactams and cephalosporins, with these being used on 31%, 56% and 13% of farms respectively. **Error! Reference source not found.** presents a s ummary of the proportions of use of the antimicrobial classes across all farms, with use given as a percentage of total AMU.

Table 2.6~~2.6~~. An outline of descriptive statistics of the variation of use between classes of antimicrobials, expressed as percentages of overall use, across all farms.

Antimicrobial Class	Range	Mean	Median
Aminocoumarin	0-11.7	2.38	0.35
Aminoglycoside	10.7-48.7	27.2	26.5
B-lactam	11.2-51	32.5	33.2
Cephalosporin	3.1-43	18.8	18.4
Fluoroquinolone	0-12.3	3.95	2.45
Lincosamide	0-3.2	0.45	0
Macrolide	0-7	1.98	1.3
Trimethoprim/Sulfonamide	0-49	11.3	8.2
Tetracycline	0-11.7	2.5	1.35

Aminoglycoside use across all farms primarily occurred in conjunction with β-lactam antimicrobials, either as an injectable solution or as an intramammary suspension in the treatment of bovine mastitis. Cephalosporin based antimicrobials were used in a range of both injectable solution and intramammary suspension based products. Calculations of AMU across farms according to mg/PCU figures demonstrated variability between farms and are presented in Table 2.7 along with an overview of herd size. Mean AMU across all farms between 2014 and 2018 was 8.31 mg/PCU. ~~with a range of 11.8 mg/PCU~~.

Table 2.7. An overview of herd descriptives detailing milking herd size and the farm's respective 5 year rolling measure of AMU (mg/PCU) entailing use for years 2014-2018.

Farm ID	Milking herd size	5 year AMU average (mg/PCU
01	230	3.14
02	220	13.65
03	280	5.85
04	210	12.97
05	80	4.76
06	14	3.1
07	85	9.07
08	230	11.88
09	74	14.91
10	100	6.73
11	226	5.44
12	220	9.48
13	42	8.52
14	234	6.1
15	156	4.59
16	10	12.71

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Figure 2.1 shows an example from one of the sixteen farms of a profile of antimicrobial use history. Profiles for all farms are included in appendices (Appendix Chapter 2). Visual assessment of these profiles suggest 31% of farms had no clear change in AMU during this time. Of the remaining 69%, 13% were considered to have as having a declining overall AMU across all classes used. Another 13% were using less cephalosporin and fluoroquinolone antimicrobials by the end of the period, but 31%, which were identified as using less cephalosporin and fluoroquinolone, featured increasing use of other antimicrobials, largely belonging to the β-lactam, aminoglycoside and trimethoprim/sulfonamide classes. Finally, 13% were using less or no fluoroquinolone only.

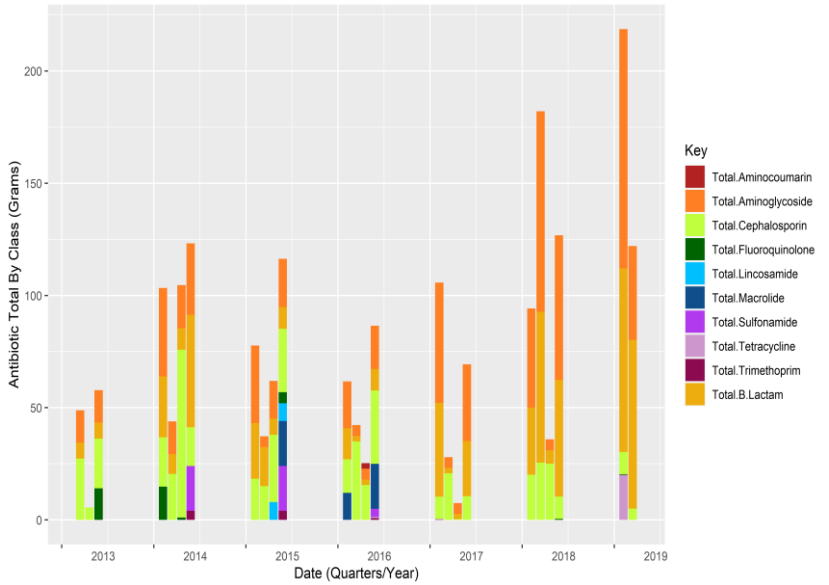

Figure 2.1**.** An example of the graphical illustration of AMU for a single farm across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

2.3.2 Antimicrobial resistance descriptive results

For the entire sampling period (August 2014 to November 2019), a total of 1163 bacterial isolates which were recovered were included in final analysis (*E. coli*; n= 448, *E. faecalis*; n= 441, *E. faecium*; n= 228, *E. durans*; n= 46). An overview of the distributions of the raw MIC data generated for each sampling date are provided in the appendices (Appendix, Chapter 2). Tables A2.1 to A2.8 ~~Table 2.7-2.14~~ provide an overview ~~of the distributions of the raw MIC data generated~~ for each sampling date for the *Enterococcus spp.* isolates tested against the range of antimicrobials included on the COMPGNF1 microdilution plate, while ~~Table 2.15-2.19~~ Tables A2.9 to A2.13 provide an overview of these data for the *E. coli* isolates tested. ~~Table 2.20-2.27~~ Tables A2.14 to A2.21 shows MIC data in the same format for *Enterococcus spp.* tested against the range of antimicrobials included on the Micronaut-S Mastitis 3 microdilution plates, while ~~Table 2.28-2.32~~ Tables A2.22 to A2.26 provide an overview of this for *E.coli* isolates tested. Also presented are the proportion of

isolates deemed to be resistant, where clinical breakpoints have been established, expressed as a percentage.

For antimicrobials tested against *Enterococcus spp*. on the COMPGNF1 microdilution plates, the most frequently observed resistance was against tetracycline, with an mean of 22.7% of isolates being deemed resistant across the eight sampling dates, with the next most common resistance being observed for imipenem. Although all isolates were deemed to be resistant to cefazolin, the intrinsic resistance to cephalosporins shown by *Enterococcus* spp. is likely to be the reason for this. Across the whole sampling period for *Enterococcus* spp., only one isolate was found to be resistant to ampicillin and none were found to be resistant to amoxicillin/clavulanic acid. For *E.* coli, the most common resistance observed was against ampicillin, with a mean rate of resistance across the five sampling periods of 5.6%, followed by tetracycline at 4.6%. February 2019 saw an increase in the levels of resistance compared to November 2018, which was followed by the lowest levels of resistance observed for *E. coli* in the following sampling period of August 2019.

For antimicrobials tested on the Micronaut-S Mastitis 3 microdilution plates, few have established clinical breakpoints (only erythromycin and penicillin for *Enterococcus* spp.; only cefoperazon for *E. coli*). MIC profiles for both bacterial species were generally stable across the sampling periods, with little change seen especially for *E. coli* isolates.

Overall, variation between sampling periods was subtle, however no clear increase in levels of AMR between the start and end of the monitoring period were found.

2.3.3 Statistical analysis

Statistical analyses were performed only for *Enterococcus* spp. as inspection of data indicated that there was no obvious effect of AMU on differences in MICs amongst *E. coli* isolates. A graphical overview of the MIC distribution for the nine antimicrobials considered in final statistical analysis is provided in Figure 2.2 (page 65) and presents the percentage of all isolates of *Enterococcus* spp. corresponding to their respective

MIC measure. Outcomes of analysis of both linear and linear mixed effects regression when considering total AMU are presented in Table 2.8~~7~~. For the individual antimicrobials considered in the analysis and respective AMU by antimicrobial class, no statistically significant relationships were observed across the majority of those investigated. However, a statistically significant relationship was found for the relationship between aminoglycoside use and MICs for gentamicin in linear regression modelling. In this instance, the relationship was found to be negative, where higher aminoglycoside use was found to be associated with lower MICs for gentamicin.

Table 2.8~~2.7~~. An overview of linear and linear mixed effects regression models of associations between total AMU by antimicrobial class and MICs of individual antimicrobials considered in analysis.

Antimicrobial class	**Antimicrobial**	**Linear regression**	**Std.Error**	**Mixed effects linear regression**	**Std. Error**
Aminoglycoside	Gentamicin	0.024*	0.351	-0.444	0.13
β-lactam	Amoxicillin/Clavulanic Acid	0.066	0.248	1.271	0.083
	Ampicillin	0.602	0.248	1.71	0.084
	Penicillin G	0.647	0.277	1.842	0.094
Cephalosporin	Cefquinome	0.994	0.522	-0.068	0.615
Fluoroquinolone	Enrofloxacin	0.57	0.261	0.56	0.262
	Marbofloxacin	0.714	0.178	0.369	0.178
Macrolide	Erythromycin	0.7	0.657	-0.218	4.79
Tetracycline	Doxycycline	0.69	0.458	-1.294	1.868

* indicates statistically significant relationship

Results from statistical analysis carried out using AMU metrics DDD (using data on lactating intramammary tubes) and DCD (using data on lactating and dry cow intramammary tubes) are shown in Tables 2.9 -2.10 respectively. In both cases, no statistically significant relationships were found between MICs in *Enterococcus* spp. and the respective AMU metrics.

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Table 2.9~~2.8~~. An overview of linear and linear mixed effects regression models of associations between AMU (as measured by DDD of lactating cow intramammary tubes) and MICs of individual antimicrobials considered in analysis.

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Antimicrobial class	**Antimicrobial**	**Linear regression**	**Std.Error**	**Mixed effects linear regression**	**Std. Error**
Aminoglycoside	Gentamicin	0.132	0.230	1.107	0.251
β-lactam	Amoxicillin/Clavulanic Acid	0.781	0.139	0.567	0.151
	Ampicillin	0.894	0.137	0.616	0.150
	Penicillin G	0.124	0.150	-1.459	0.154
Cephalosporin	Cefquinome	0.151	0.327	-1.230	0.354
Fluoroquinolone	Enrofloxacin	0.498	0.262	-0.623	0.270
	Marbofloxacin	0.528	0.179	-0.630	0.180
Macrolide	Erythromycin	0.477	0.335	0.715	0.348
Tetracycline	Doxycycline	0.601	0.421	0.824	0.458

Table 2.10~~2.9~~. An overview of linear and linear mixed effects regression models of associations between AMU (as measured by DCD of lactating and dry cow intramammary tubes) and MICs of individual antimicrobials considered in analysis.

Antimicrobial class	**Antimicrobial**	**Linear regression**	**Std.Error**	**Mixed effects linear regression**	**Std. Error**
Aminoglycoside	Gentamicin	0.308	0.218	0.852	0.230
β-lactam	Amoxicillin/Clavulanic Acid	0.743	0.131	0.705	0.134
	Ampicillin	0.711	0.129	1.116	0.137
	Penicillin G	0.249	0.143	-1.072	0.147
Cephalosporin	Cefquinome	0.344	0.311	-0.894	0.326
Fluoroquinolone	Enrofloxacin	0.681	0.247	-0.372	0.253
	Marbofloxacin	0.638	0.168	-0.469	0.169
Macrolide	Erythromycin	0.427	0.316	0.817	0.324
Tetracycline	Doxycycline	0.335	0.396	0.927	0.409

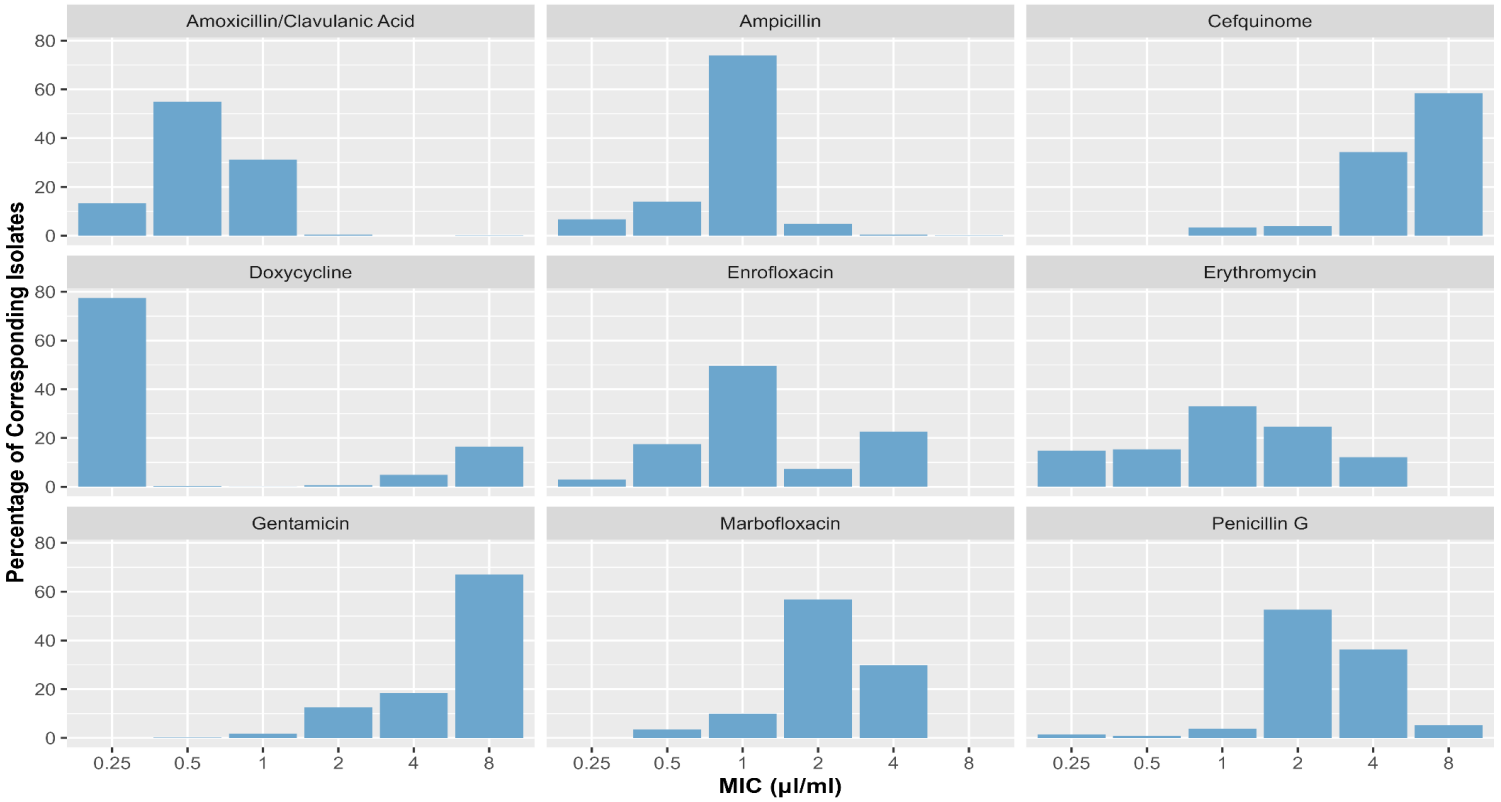

Figure 2.2. Overview of the distribution of MICs for the nine antimicrobials included in the final analysis for *Enterococcus* spp. across the entire sampling period (August 2014 – August 2019), shown as a percentage of isolates corresponding to their respective MIC value (n = 715).

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2.4 Discussion

The aim of this Chapter was to identify the extent to which AMU was associated with trends in antimicrobial susceptibilities of sentinel bacterial species within and between study group herds. This study provided an opportunity to conduct long term monitoring within a closed island population without the potential for resistant bacteria or resistance related genes to enter from external livestock hosts.

Collation of data from veterinary sales records highlighted patterns of AMU within and between farms. Data showed that aminoglycosides and β-lactam antimicrobials were the most frequently used across the study population, with cephalosporins also accounting for a high proportion of use. Research conducted by Hyde et al., (2017) reported that the most commonly used antimicrobial products were β-lactams and aminoglycosides, representing 42.8 % and 20.9% of ingredient mass (mg) respectively. This compares with an average use of β-lactam and aminoglycosides antimicrobials for farms representing this current study of 32.5 % and 27.2 % respectively. Fluoroquinolone use along with third and fourth generation cephalosporin use accounted for an overall low proportion of use in terms of antimicrobial active ingredient in findings presented by Hyde et al., (2017) whereas use of these antimicrobial classes was found to be higher on average on farms in the current study. For comparison, the average AMU 22.11 mg/PCU according to the 2017 study, while the average for all farms involved in this study across a period of five years (2014-2018) was 8.3 mg/PCU.

Where data showed decreases in cephalosporin and fluoroquinolone use, this was generally associated with increases in use of other antimicrobial classes, largely aminoglycosides and β-lactams. Investigation of AMU on Dutch dairy farms between 2005 and 2012 (Kuipers et al., 2016) reported that the use of third and fourth generation cephalosporins and fluoroquinolones fell from 18% of overall use to only 1%. This reduction however brought about an increase in use of penicillin and other β-lactam products as well as broad spectrum products such as trimethoprim/sulfonamide combinations. Given that the reduction in use of one

antimicrobial class may lead to increased use of another, continued surveillance of antimicrobial susceptibilities of these antimicrobial classes will be critical.

The lack of statistically significant relationships between AMU, across the three metrics considered, and MICs was an unexpected outcome, given the widely reported correlation between AMU and AMR (Chantziaras et al., 2014). ~~(Chantziaras et al, 2014).~~ This may be due to a relatively small study population (n=16) with relatively little variation in antibiotic susceptibilities across the monitoring period in both sentinel bacterial species. In research carried out in South Korea investigating changes in antimicrobial susceptibilities of Gram-negative bacteria including *E. coli*, Nam et al., (2009) reported no significant change in the incidence of AMR amongst bacteria between 2003 and 2008. A similar study from South Korea looking at the antimicrobial susceptibilities of *E.coli* between 2012 and 2015 also found no significant changes in the incidence of AMR during this time (Tark et al., 2017). Similarly with the research outlined in this Chapter, these longitudinal studies considered AMR over an extended period of time, however, AMU was not considered.

Although there has been a great deal of work done to quantify AMU on dairy farms over time as well as longitudinal studies considering AMR, investigations of the associations between AMU and AMR is less common. Research by Saini et al., (2013) which followed up on previous work quantifying AMU, reported that there was an association between AMR in *E. coli* isolated in bovine mastitis cases and antimicrobials commonly used on Canadian dairy farms. This contrasts with the findings presented in this Chapter, where it was concluded that variability in the MIC profiles of *E. coli* isolates tested was too low to meaningfully consider the role of AMU. These authors, however, did report the existence of negative associations between AMU and AMR amongst certain antimicrobial classes. A similar finding was made in this Chapter, but amongst *Enterococcus* spp. rather than *E. coli*.

The negative association between aminoglycoside use and lower MICs for gentamicin for *Enterococcus* spp. is difficult to ascertain. Intrinsic, sporadic and acquired resistances to aminoglycosides have been identified for *E. faecalis* and *E. faecium*, however, high level intrinsic resistances to gentamicin have not been determined

(Hollenbeck and Rice, 2012). Gentamicin had been included in analysis as some variation across the entire sampling period was identified. The negative association found may indicate the existence of low level intrinsic resistance which may have therefore contributed to this result. Additionally, low variation in terms of AMU and AMR identified across this study population may have resulted in poor linear regression model performance. Therefore the questionable outcome may be explained by measurement errors rather than biologically.

As found in this Chapter and elsewhere in literature, aminoglycosides constitute a large proportion of AMU on dairy farms. Additionally, relationships have been found between higher levels of AMU and increased AMR amongst *Enterococcus* spp. of animal origin (Hershberger et al., 2005). Therefore, the role of *Enterococcus* spp. as a potential reservoir of AMR related genes, including intrinsic aminoglycoside resistance (Kang et al., 2021) warrants continued monitoring due to the potential for dissemination via horizontal gene transfer (von Wintersdorff et al., 2016).

From their identification of negative AMU/AMR associations, Saini et al., (2013) postulated that, despite being unconvincing in biological terms, other factors may be responsible in the determination of AMR other than AMU. This therefore highlights interest in other areas which may influence AMR at the dairy farm level.

2.5 Study Limitations

Despite capturing the majority of farms making up the target population, a relatively small sample size of sixteen farms may have been limiting. This may have impacted the ability to identify significant differences between herds in terms of their AMU, however, the fact that six years of antimicrobial sales records were accessed and analysed may offset this.

Additionally, the nature of AMU data capture may also have been a limitation. Sales records identified only what antimicrobial products were being purchased at a given time, but this doesn't mean that the entire product was used at once. Records of medicine administration recorded in the farm medicine book may have provided

greater detail of how much antimicrobial product was being used at a given time and to what kind of animal (youngstock vs adult dairy cow). This would have been especially true for dry cow therapy, were product is generally sold in a large quantity.

Finally, the lack of historical *E. coli* isolates was a further limitation. As *E. coli* was difficult to revive from frozen bulk tank samples from August 2014 – August 2017, the period of time to investigate potential changes in MICs occurring within *E. coli* isolates over time was much shorter than that for *Enterococcus* spp. Therefore, some patterns in MICs, and therefore potential associations with AMU, may have been missed.

2.6 Conclusion

In conclusion, despite the breadth of data considered as part of this longitudinal study, statistically significant associations between higher AMU and higher levels of resistance, as measured by MICs in sentinel bacteria, were not found. This was in spite of a recognised biological basis of the selection for resistance created by AMU. However, it was found that, when compared with mainland UK dairy herds, the study population recruited as part of this research used less antimicrobials, which could pose a potential explanation for these findings. The existence of a negative association as identified between aminoglycoside use and MICs for gentamicin may be explained by intrinsic aminoglycoside resistance amongst *Enterococcus* spp. or through measurement error, but further investigation may be warranted to fully explain this finding.

The outcomes identified from this study led to an interest in other areas which may influence AMR in the dairy farm environment. In addition to AMU, the influence of farm management system (conventional vs organic) on AMR has been acknowledged and it is suggested that management is the most important factor related to resistance after AMU (Murphy et al., 2018). The farm visits which were briefly alluded to in this chapter were used as an opportunity to gather data related to farm management. An investigation into the influence of a number of farm management practices on antimicrobial susceptibilities is presented in Chapter 3 of this thesis.

Chapter 3; Cross sectional study of farm management and antimicrobial resistance

3.1 Introduction

From analysis of associations between AMU and bacterial MICs in Chapter 2, a lack of statistically significant relationships were found for almost all antimicrobial classes. In addition to AMU, the influence of farm management system (e.g. conventional vs organic) on AMR has been acknowledged. It has been suggested that management is the most important factor related to resistance after AMU (Murphy et al., 2018). These authors noted that although conventional and organic systems were identified as a point of importance, these systems may represent a range of practices relating to farm management, such as housing, biosecurity and farm density. Such factors may play an important role in the emergence of AMR, either through direct associations or indirectly, by encouraging increased AMU. Therefore, to fully understand AMR on-farm, simultaneous investigation of both AMU and general farm management policies is needed.

The aim of this Chapter was to evaluate the associations between farm management practices and the resistances of sentinel bacteria in bulk tank milk. Farm data were collected during face-to-face interviews from two dairy herd populations with resistances measured according to MICs of *Enterococcus* spp. and *E. coli*. Dairy herd populations were categorised as study group 1 and study group 2. Data constituting study group 2 were collected prospectively, specifically for this research. Study group 1 consisted of historical data and were included to allow comparison with findings from study group 2. Methods regarding bacteriology and susceptibility testing were not identical between studies since the two were independent and carried out at different times. The principles of data collection, sample handling and analyses were the same for both studies.

3.2 Materials and methods

3.2.1 Farm recruitment

Study group 1 consisted of 125 dairy farms located across England and Scotland used in a previous research study (Bradley et al., 2018). Farms were recruited on the basis of bedding material used in dairy cow housing; recycled manure solids (RMS), fresh sand or sawdust. The aim was to recruit a minimum of 40 farms using either of these materials, with farmers being approached via contacts made previously by the research team, veterinarians and participating farmers. Farms to be recruited were additionally matched according to milking method (conventional or automated) and geographic location (East/West UK). Detailed recruitment of farms is described by Bradley et al., (2018).

Study group 2 consisted of the sixteen dairy farms recruited for the study described in Chapter 2, where farm recruitment has been explained (2.2.2).

3.2.2. Questionnaire design

Questionnaires were developed to capture a broad range of management practices potentially associated with AMR within a previous 12 month period. The questionnaire intended for use in study group 1 had been designed for prior research purposes. The questionnaire featured mainly multiple choice, yes or no and closed questions. Firstly, the questionnaire addressed basic farm demographics in terms of herd size, number of cows in milk and total milk sales for the previous year. The second main section of the questionnaire was dedicated to the grouping and housing of adult dairy cows (both lactating and dry) and the types of bedding used within each area of housing. Observations on cubicles were also made regarding; whether they featured mats or mattresses, a bedding retainer and features related to cubicle bedding. The subsequent section addressed the farm's milking procedures; milking frequency, parlour type, clusters and cluster disinfection, teat and parlour hygiene. Data surrounding dairy cow nutrition and herd health records were also captured.

Design of the questionnaire for study group 2 was based on that used for study group 1. Additional information relating to some sections of the previous questionnaire were added, along with novel areas for data capture which weren't previously considered. The first section considered details of livestock demographics, movement of animals to and from the farm and on farm fertility management. The following section considered areas of the milking procedure in terms of hygiene of teats and that of the milking parlour. The third main section of the questionnaire focused on dry cow and mastitis management, vaccinations, use of anthelmintics and herd health record keeping. The fourth section focused on the management of dairy cow housing and bedding. The fifth section addressed calf management and final section of the questionnaire focused on the farm environment; use of chemicals and detergents, slurry and waste bedding management and the interface between the farm and wildlife. The questionnaire also provided for the farmer to comment on any changes in management routines which occurred within the previous 12 months, ensuring this information was also captured.

3.2.3 Data collection

3.2.3.1 Questionnaires

For both study groups, farm management data were collected by means of a face to face interview with farmers during dedicated farm visits. Data from study group 1 were collected during farm visits carried out by five members of a dairy consultancy organisation between December 2014 and March 2015 with each farm being visited once by one consultant. Observational data were also recorded. For study group 2, questionnaires were conducted during a single farm visit between January and April 2019. All questionnaire data were collated in a spreadsheet (Microsoft Excel, Microsoft Corporation, 2016). Data were checked for outlying or implausible values, but none requiring removal were identified. Questions which resulted in categorical data were given numeric codes for the purpose of analysis.

3.2.3.2 Antimicrobial use data

In addition to farm management, AMU data was also considered in the overall analysis with farm management data for study group 2. Collection of this data has already been described in Chapter 2 (2.2.4.1). For the purposes of analysis, AMU data between January 2018 and April 2019 were used. As the questionnaire had taken into account management practices for the previous year, this allowed AMU data to align with this time period. AMU data to be included in final analysis were calculated on a per cow basis, taking into account the herd size of each farm.

3.2.3.3 Study group 1 bacteriology

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

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

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

When ready for AST, bacterial isolates were pure plated from the stored glycerol beads onto Columbia (5% sheep blood) agar and incubated for 18-24 hours at 37°C. MICs were determined using a VITEK® 2 (Biomerieux; Basingstoke UK) according to manufacturer's instructions. VITEK® 2 AST GN65 and GP76 cards were used for determining *E. coli* and *Enterococcus* spp. MICs respectively.

All data were entered into a spreadsheet (Microsoft Excel, Microsoft Corporation, 2016). Isolates were determined as being either susceptible or resistant according to clinical breakpoints established by the Clinical and Laboratory Standards Institute (CLSI). Where CLSI breakpoints were not available, interpretative criteria provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used.

3.2.3.4 Study group 2 bacteriology

Bacterial isolates to be included in analysis for study group 2 had already been processed as described previously (Chapter 2; 2.2.5.1 and 2.2.5.2). MIC data for bulk tank milk samples delivered to the laboratory for processing between August 2018 and November 2019 were included in the current analysis. The selection of these data allowed for a better consideration of bacterial MICs for a period prior to and following the carrying out of farm management questionnaires (January – April 2019).

3.2.4 Farm resistance 'score'

MIC data for study group 1 and selected data for study group 2 were used to generate an overall resistance score for each bacterial species for each farm. The first step in this process has been described previously (Chapter 2; 2.2.5.3), whereby a mean standardised MIC was calculated for each antimicrobial tested against both *Enterococcus spp.* and *E. coli* for each farm. Subsequently, these means were used to calculate the mean of all antimicrobials, across all isolates across all farms. For study group 1, a mean of standardised MIC values for antimicrobials tested against both bacterial species was attributed to the single bulk tank milk sample collected from each farm between December 2014 and March 2015. For study group 2, the mean standardised MIC for antimicrobials tested against both sentinel bacterial species across six samples taken from each bulk tank sample obtained between August 2018 and November 2019 was used to provide an overall resistance score. Antimicrobials which were included in final analysis for study group 1 *Enterococcus* spp. and *E. coli* are shown in Table 3.1 and Table 3.2 respectively. The antimicrobials included in analysis for study group 2 have been outlined previously (Chapter 2, Tables 2.4 and 2.5). For both study groups, this overall farm mean MIC was used as the outcome variable to evaluate the impact of farm management and AMU (study group 2 only) on MICs for *Enterococcus* spp. and *E. coli*.

Table 3.1~~3.1~~. Antimicrobials included on the AST GP76 plate which were selected for analysis, with their respective range of tested concentrations

Antimicrobial	Concentration Range (µg/ml)
Benzylpenicillin	0.12 – 64
Chloramphenicol	4-64
Enrofloxacin	0.5 – 4
Erythromycin	0.25 – 8
Nitrofurantoin	16 – 512
Tetracycline	1 – 16
Trimethoprim/Sulfamethoxazole	10 (0.5/9.5) – 320 (16/304)
Vancomycin	0.5 – 32

Table 3.2~~3.2~~. Antimicrobials included on the AST GN65 plate which were selected for analysis, with their respective range of tested concentrations

Antimicrobial	Concentration Range (µg/ml)
Amikacin	2 – 64
Amoxicillan/Clavulanic Acid	2/1 – 32/16
Ampicillin	2 – 32
Cefalexin	4 – 64
Cefovecin	0.5 – 8
Cefpodoxime	0.25 – 8
Ceftiofur	1 – 8
Chloramphenicol	2 – 64
Enrofloxacin	0.12 – 4
Gentamicin	1 – 16
Imipenem	1 – 16
Marbofloxacin	0.5 – 4
Nitrofurantoin	16 – 512
Piperacillin	4 – 128
Polymyxin B	0.25 – 16
Tetracycline	1 – 16
Tobramycin	1 – 16
Trimethoprim/Sulfamethoxazole	20 (1/19) – 320 (16/304)

3.2.5 Data analysis

Following data collation and prior to the commencement of statistical analysis procedures, all questionnaire datasets were checked for the frequency of each category for each explanatory variable. Where particular categories accounted for a small proportion of the overall category for a given explanatory variable, the variable was removed from the dataset as this could reduce the power of the regularised regression procedure. A finalised set of questions and responses which were used as part of statistical analysis are included in appendices (Appendix Chapter 3).

Due to the large number of potential explanatory variables relative to the number of observations (herds), regularised regression with stability selection was conducted for inference (Zou and Hastie, 2005; Meinshausen and Bühlmann, 2010) to avoid overfitting. Explanatory variables were coded as numeric or categorical and numeric covariates were standardised to a common scale, by subtracting the mean and dividing by twice the standard deviation, as previously reported (Gelman, 2008).

3.2.5.1 Regularised regression

Regularisation was carried out using a linear elastic net regression model with a continuous outcome using the "glmnet" and "caret" packages (Friedman et al., 2010; Kuhn et al., 2018) within R (RStudio Team, 2020). Farm resistance 'scores' were defined as the outcome variable and farm management practices as the explanatory variables. Elastic net regression combines the effects of ridge and lasso regression (Zou and Hastie, 2005). Penalised maximum likelihood was used to fit models with a cyclical coordinate descent algorithm to conduct parameter estimation via algorithms which solve the equation through cyclical coordinate descent (Friedman et al., 2010). Elastic net models constructed for both study groups took the following form;

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

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

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

To estimate covariate stability and P-values, a bootstrapping procedure was undertaken to ensure robust estimation of model parameters (Hastie et al., 2015; Lima et al., 2020). In brief, this comprised using a bootstrapping procedure to rerun elastic net models 500 times. Model parameters from each bootstrapped sample were stored in a matrix and used for inference. Final inference was based on two

main outcomes - parameter stability and a bootstrapped P-value. Parameter stability refers to the percentage of times that a particular variable was selected in the model across the 500 bootstrap samples; the higher the percentage, the less likely the covariate is to be a false positive result (Meinshausen and Bühlmann, 2010). The 'Bootstrap P value' (BPV) was calculated as the minimum proportion of (non-zero) coefficient values to one side of zero. That is, if a covariate was selected in the model in 400 of the bootstrap samples and 390 of these had a value either greater or less than zero, then the Bootstrap P value would be (400-390)/400 = 0.025. Covariates were selected in the final model and deemed 'significant' when both BPV < 0.05 with a high covariate stability. These thresholds were identified by plotting stabilities against significance and are shown in Figure 3.1, Figure 3.2 andFigure 3.3. *Enterococcus* spp. and *E. coli* model stabilities for study group 1 were defined as ≥80% and ≥75% respectively, while the *Enterococcus* spp. stability for study group 2 was defined as ≥55%.

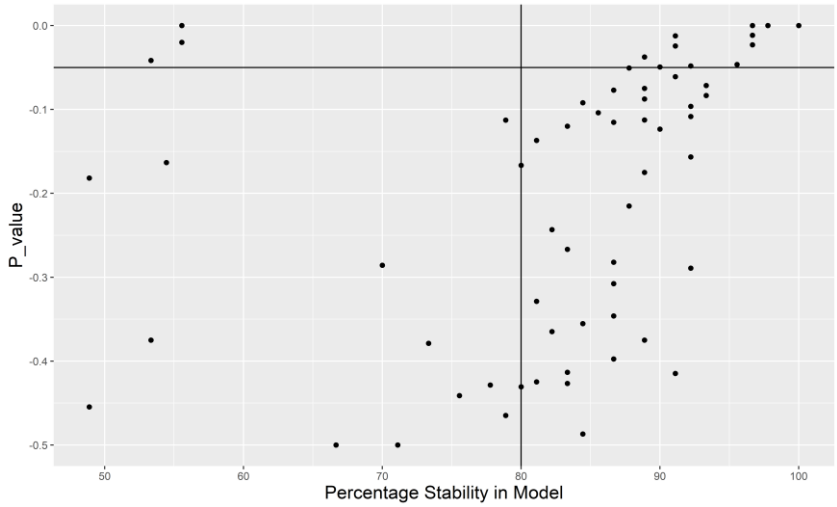

Figure 3.1. Plot of stability against bootstrapped P-value used to identify covariates of importance in the final elastic net model produced for *Enterococcus* spp. in study group 1 farms; covariates in the top right hand quadrant were selected in the final model.

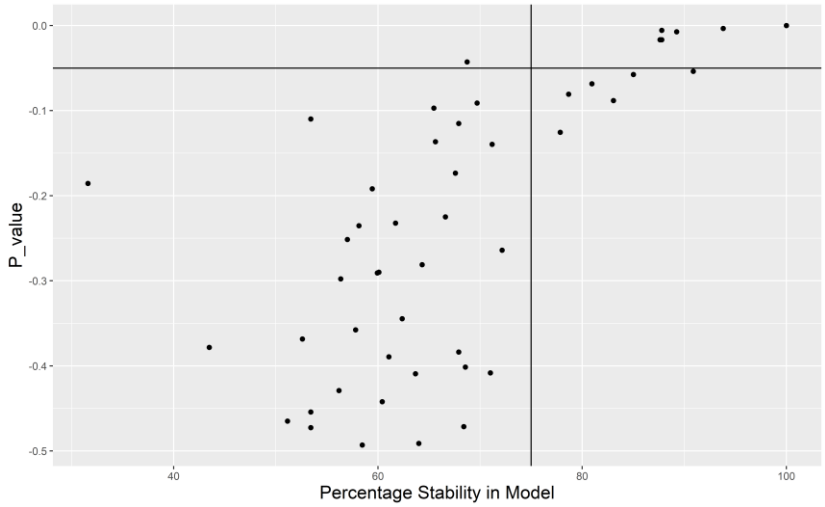

Figure 3.2. Plot of stability against bootstrapped P-value used to identify covariates of importance in the final elastic net model produced for *E. coli* in study group 1 farms; covariates in the top right hand quadrant were selected in the final model.

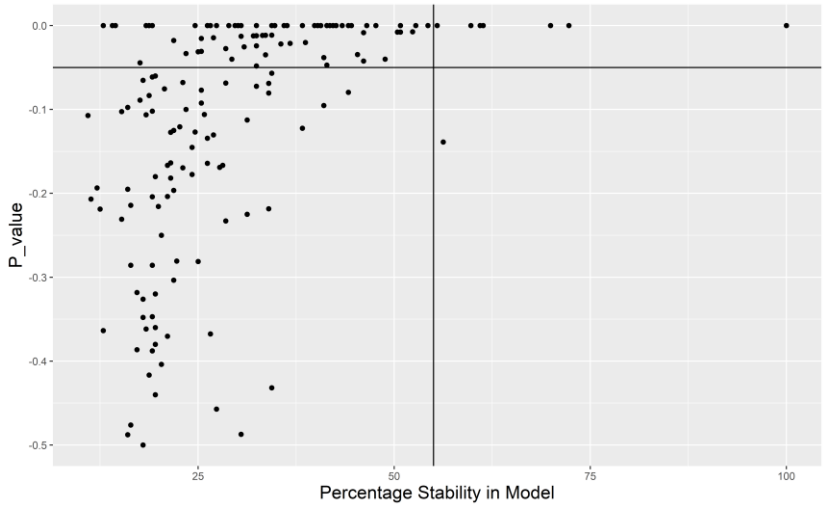

Figure 3.3. Plot of stability against bootstrapped P-value used to identify covariates of importance in the final elastic net model produced for *Enterococcus* spp. in study group 2 farms; covariates in the top right hand quadrant were selected in the final model.

3.3 Results

3.3.1 Population characteristics

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

		Number of isolates								
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**>4**	**8**	**>**
Amoxicillin/Clavulanic Acid	0		37	217	108	2	-		1	
Ampicillin	0.3		18	49	278	17	2		-	
Cefquinome [a]	-				3	10	135		92	1
Doxycycline	2.2		284	2	-	1	21		49	
Enrofloxacin [a]	-	-	8	65	199	24	43	26		
Erythromycin	7.1	37	18	61	132	74	17	26		
Gentamicin [a]	-		-	-	5	31	62		91	1
Marbofloxacin [a]	-	-	-	16	34	226	66	23		
Penicillin G	1.92	2	-	-	16	198	125		17	

3.3.2 Minimum inhibitory concentration distributions

For study group 1, final analysis included 171 *E. coli* isolates and 293 *Enterococcus* isolates (*E.faecalis;* n=93, *E.faecium; n*=107, *E.durans; n=93).* Data pertaining to the percentage of isolates deemed resistant and the distribution of MICs are presented in Table 3.3, Table 3.4 and Table 3.5.

For study group 2 data (sampling period August 2018 – November 2019), 365 *Enterococcus* spp. (*E.faecalis;* n=249, *E.faecium; n*=97, *E.durans; n=19*) and 451 *E. coli* isolated from milk samples were included in final analysis. The percentage of these isolates deemed resistant alongside MIC distributions for this dataset are presented in

Table 3.6 and Table 3.7

Table 3.3~~3.3~~. Distribution of the MICs of *Enterococcus spp.* isolated (n= 293) from study group 1 farms (n=94) alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)												
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
Benzylpenicillin	3.1	59	60	24	24	91	15	11	9	-	-			
Chloramphenicol	3.4						135	148	-	-	10			
Enrofloxacin [a]	-			175	28	33	57							
Erythromycin	5.1		130	24	30	88	6	15						
Nitrofurantoin	13								-	127	128	25	13	-
Tetracycline	32.4				192	6	-	-	95					
Vancomycin	0			208	59	26	-	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table 3.4~~3.4~~. Distribution of the MICs of *E. coli.* isolated (n=171) from study group 1 farms (n=87) alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)												
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
Amikacin	0					168	3	-	-	-	-			
Amoxicillan/Clavulanic Acid	3.5					123	31	11	5	1				
Ampicillin	13.5					86	36	26	-	23				
Cefalexin	1.2						6	130	33	-	2			
Cefovecin	2.3			112	53	2	-	4						
Cefpodoxime	0		145	16	6	-	4	-						
Ceftiofur [a]	-				167	1	-	3						
Chloramphenicol	4.7					50	60	40	13	-	8			
Enrofloxacin [a]	-	169	-	-	2	-	-							
Gentamicin [a]	-				168	-	3	-	-					
Imipenem	0.6				170	-	-	1	-					
Marbofloxacin [a]	-			169	2	-	-							
Nitrofurantoin	0.6								125	37	8	1	-	-
Piperacillin	11.1						147	3	2	-	3	16	-	-
Polymyxin B	0.6		5	116	40	9	1	-	-					
Tetracycline	14				146	1	-	-	24					
Tobramycin	1.8				168	-	-	-	3					

[a] Clinical breakpoints for resistance not defined

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Table 3.5~~3.5~~. Distribution of MICs of trimethoprim/sulfamethoxazole of *Enterococcus spp.* (n=293) and *E. coli* (n=171) isolates from study group 1 farms. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)					
Species	**% of isolates deemed resistant**	**0.5/9.5**	**1/19**	**2/38**	**4/76**	**8/152**	**16/304**
Enterococcus spp. [a]	-	290	-	-	-	2	1
E. coli	0		167	2	-	-	-

[a] Clinical breakpoints for resistance not defined

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Table 3.6~~3.6~~. Distribution of the MICs of Enterococcus spp. isolated (n=365) from study group 2 farms (n=16) alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)								
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**>4**	**8**	**>8**
Amoxicillin/Clavulanic Acid	0		37	217	108	2	-		1	-
Ampicillin	0.3		18	49	278	17	2		-	1
Cefquinome [a]	-				3	10	135		92	124
Doxycycline	2.2		284	2	-	1	21		49	8
Enrofloxacin [a]	-	-	8	65	199	24	43	26		
Erythromycin	7.1	37	18	61	132	74	17	26		
Gentamicin [a]	-		-	-	5	31	62		91	176
Marbofloxacin [a]	-	-	-	16	34	226	66	23		
Penicillin G	1.92	2	-	-	16	198	125		17	7

[a] Clinical breakpoints for resistance not defined

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Table 3.7~~3.7~~. Distribution of the MICs of *E. coli.* isolated (n=451) from study group 2 farms (n=16) alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)							
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**
Amoxicillin/Clavulanic Acid	1.6		-	-	4	138	259	43	7
Ampicillin	6		1	-	37	223	158	6	26
Cefquinome [a]	-				444	1	2	3	1
Doxycycline	3.3		-	11	156	253	12	4	15
Enrofloxacin [a]	-	443	6	1	-	1	-		
Gentamicin	1.1		1	218	204	23	-	-	5

[a] Clinical breakpoints for resistance not defined

3.3.3 Statistical models

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

3.3.3.1 Study group 1; *Enterococcus* spp.

Results of the final model for management factors associated with *Enterococcus* spp. MICs for study group 1 are presented in Table 3.8. Covariates selected in the final model related to the size of milking parlour, farm location, use of automatic milking systems and practices associated with bedding materials. Farms with parlours containing between 13-24 units and those between 25 and 36 milking units had higher MICs than farms with smaller parlours of ≤12 milking units. In terms of geographic location, farms in the north west of England had higher *Enterococcus* spp. MICs when compared to farms elsewhere in the country. Farms with automated milking systems had higher MICs than those where cows were milked conventionally. Practices associated with cubicle bedding were selected in the final model with farms using recycled manure solids (RMS) having increased MICs compared to those using sawdust. A decreased frequency of cubicle bedding was associated with lower MICs in *Enterococcus* spp.

3.3.3.2 Study group 1; *E. coli*

Results of the final model for management factors associated with *E. coli* MICs for study group 1 are presented in Table 3.9. Bedding of cubicles once daily was associated with significantly lower MICs for *E. coli* compared to farms that bedded cubicles twice daily. Significantly lower MICs were identified on farms that did not use bedding conditioner materials on cubicles compared with farms that did. Milk yield was found to be important; increasing yields (litres produced per cow per

year) were associated with significantly increased MICs. Milking preparation procedures involving teat brushing ~~resulted~~ were associated with ~~in~~ significantly increased MICs, whereas the wiping of teats with dry cloths or towels resulted in significantly reduced MICs compared to the use of pre-milking teat disinfection without brushing. Milking system was again found to be important, with farms using automated milking systems being associated with significantly lower MICs than those where cows were milked in a conventional parlour.

3.3.3.3 Study group 2; *Enterococcus* spp.

Results of the final model for management factors and antibiotic use associated with *Enterococcus* spp. MICs for study group 2 are presented in Table 3.10. The presence of a slurry store on farm was found to be important; farms without slurry stores had significantly lower MICs than those with a store. Farmers who purchased antimicrobials online had *Enterococcus* spp. isolated from bulk milk with significantly higher MICs than those who purchased medicines from their veterinary practice only. Several factors relating to cubicle management were found to be important. Farms where hydrated lime was used on cubicles as an antibacterial product resulted in a significantly higher MIC than those that did not use any antibacterial products. For farms where bulls used for breeding were reared on farm rather than being borrowed or purchased, significantly lower mean MICs were identified. Farms that did not practice 'natural' drying off (i.e. always used either antibiotic therapy or teat sealants) had a significantly higher mean MIC than those farms where natural drying off was practiced.

Antimicrobial classes identified from veterinary sales records were; aminocoumarin, aminoglycoside, β-lactam, cephalosporin, fluoroquinolone, lincosamide, macrolide, sulfonamide/trimethoprim and tetracycline. The use of two classes of antimicrobials were found to be of importance in the model; higher levels of β-lactam and fluoroquinolone usage were associated with statistically significant higher MICs in *Enterococcus* spp.

Table 3.8~~3.8~~. Final elastic net regression model for farm management practices, in order of descending covariate stability, associated with changes in MIC of *Enterococcus* spp. from bulk tank milk samples for study group 1 (n=94 farms). Covariate stability threshold for variable selection was >80%.

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

Table 3.9~~3.9~~. Final elastic net regression model for farm management practices, in order of descending covariate stability, associated with changes in MIC of *E. coli* from bulk tank milk samples for study group 1 (n=87 farms). Covariate stability threshold for variable selection was >75%.

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

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

Table 3.10~~3.10~~. Final elastic net regression model for farm management practices and antimicrobial use, in order of descending covariate stability, associated with changes in MIC of *Enterococcus* spp. from bulk tank milk samples for study group 2 (n=16 farms). Covariate stability threshold for variable selection was >55%.

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

3.4 Discussion

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

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

The importance of slurry in the context of antimicrobial susceptibilities was identified for study group 2. In this study we found that on farms where there were no slurry stores, there were lower MICs compared to farms where stores were in use. This refers to the storage in above ground structures of animal waste during a period

when spreading of slurry on land is prohibited between October and February due to environmental concerns. Outside of this period, slurry may be spread on farmland. On farms without these storage facilities, slurry was contained in tanks underneath cattle housing, which is the most typical form of storage on dairy farms. The above ground stores rather facilitated an increased volume of longer-term storage. Some farms also indicated that where solid floor housing sheds existed, specific tanks existed for the collection of dirty water and yard runoff, which would later be spread onto farm land. Farm animal manure has been identified as a significant reservoir of antimicrobial compounds, resistant bacteria and antibiotic resistant genes (Heuer et al., 2011). Slurry storage is noteworthy as it facilitates an environment with the potential to encourage AMR to emerge and spread (Lanyon et al., 2021). Baker et al., (2016) evaluated the role of slurry storage in AMR via mathematical modelling. The authors reported that the proportion of bacteria showing AMR characteristics increased throughout the storage period as a result of horizontal gene transfer and by selection of resistant genes. Our study presents results similar to previous findings and suggest the role of slurry storage may be important in contributing to increased MICs on farm. Larger volumes of slurry in long-term storage throughout the slurry spreading prohibition period would facilitate the exchange of AMR related genes within the bacterial population. Importantly, the spreading of stored slurry onto land used for grazing and silage may represent a potential route for transmission of resistant organisms to dairy cows and perpetuate their existence in the farm environment.

Commented [DM6]: Explanation of point about slurry storage

Commented [DM7]: Further as above

Results from study group 2 indicated that the use of ~~antibacterial~~ antimicrobial materials on cubicle bedding to be important with regards to *Enterococcus* spp. isolated from farms in this group. The use of hydrated lime was associated with increased MICs, whereas decreased MICs were seen on farms that did not use any antibacterial products on cubicles. Additionally, as identified for *Enterococcus* spp., the use of antibacterial bedding conditioners (including hydrated lime) in study group 1 was associated with increased MICs in *E coli*. It has been reported that the use of antibacterial materials, such as lime based products, significantly reduce bacterial counts in bedding and on cow teats (Janzen et al., 1982; Paduch et al., 2013). The

association found in this study, between the use of antibacterial products on bedding and increased MICs, may be a result of an increased selection pressure on the bacterial populations present in cubicle bedding. This may inadvertently encourage selection for genes giving rise to increased MICs. However, the bacterial mechanisms for such gene selection in this context are unclear and warrants further investigation. Furthermore, there may be the possibility of reverse causation occurring in this instance. Hydrated lime may be being used to address already existing mastitis problems, which may in itself be contributing to higher MICs through increased AMU. However, as previously considered, the cross-sectional nature of this study means that only associations are identified and causality cannot be attributed.

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

Practices relating to the management of cubicles and bedding were associated with increases in MICs in *Enterococcus* spp. and *E. coli* isolates in study group 1. Here, the practice of less frequent bedding application on cubicles was associated with lower MICs. However, an overview of the data shows an association between the type of bedding material used in study group 1 and it's application frequency. Therefore, the type of bedding material used may be of greater importance compared to how often fresh material is laid down on cubicles. Additionally, there were higher MICs seen on farms that used recycled manure solids as a bedding material and this may align with the increased MICs associated with slurry storage seen in study group 2.

Furthermore, it has been reported that there were significantly higher bacterial counts in RMS bedding, when compared with sawdust or sand (Bradley et al., 2018). Within a larger population of bacteria, there may be more variability of genetic materials (as well as potential for gene transfer) and an increased chance for mutations to appear in the population. The constant recycling of manure solids, despite processing methods designed to reduce the bacterial load, may help to perpetuate this. RMS bedding materials have been found to promote growth of environmental bacteria, namely *Klebsiella pneumonia*, and to a lesser extent, *E. faecium* (Godden et al., 2008). The issue of AMR with regards to RMS due to the presence of antimicrobial residues and resistance genes has been noted, with varying levels of success across methodologies aiming to reduce their load in RMS materials (Wallace et al., 2018; Zhang et al., 2020). Our results however suggest that the increase in MICs in sentinel bacteria associated with the use of RMS should be an important consideration in its use.

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

possible that differences in antimicrobial treatment application between farms could be a driver for contrasting resistance patterns. These findings suggest that type of milking system could be important in relation to AMR and highlights this as an area for future consideration.

Purchasing of antimicrobial products by farmers in study group 2 was also found to be associated with differences in MICs of *Enterococcus* spp. Here, purchase from an online supplier (in addition to their veterinarian) was associated with higher MICs than those who purchased medicines from their veterinarian only. In the UK, antimicrobial medicines require a veterinary prescription, and best practice concerning this has been widely promoted. Despite this, it has been claimed that farmers will frequently diagnose sick animals themselves and administer antimicrobials in the absence of a veterinarian (Jones et al., 2015). Recent work has investigated the behaviours of veterinarians and farmers with regards to antimicrobial stewardship in the UK. It was found that both had a good understanding of the importance of responsible AMU, but there was a conflict between restricting use and maintaining health and welfare through antimicrobial administration. Additionally, it was found that veterinarians sometimes felt an obligation to prescribe antimicrobials due to an uncertainty around diagnosis and to meet the demands of the farmer for treatment for a sick individual (Golding et al., 2019). In study group 2, purchase of antimicrobials from an online supplier required a veterinary prescription. Information surrounding the prescription process was not captured so it is hard to ascertain the level of veterinary involvement in online purchase. However, reduced input in the diagnosis and administration of antimicrobials on the part of the veterinarian may have resulted in a level of antimicrobial overuse, thus contributing to increased MICs in *Enterococcus* spp. in study group 2 farms. Given this, in future AMU and AMR research in the context of dairy, it may be important to consider the role of the veterinarian in the prescribing and administration of antimicrobials on farm.

Commented [DM8]: Some discussion around results regarding antimicrobial purchase

The collection and collation of AMU data for farms in study group 2 helped to further highlight the importance this has for AMR at the dairy farm level. It was shown that

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

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

3.5 Study Limitations

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

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

3.6 Conclusions

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

The identification of associations highlights the value of monitoring antimicrobial susceptibilities of sentinel bacteria isolated from bulk tank milk, which has been argued as a key indicator of the whole herd population and its environment. Continued monitoring will help to further inform and direct future policy relating to antimicrobial resistance in the dairy industry. Convenient laboratory methods will be

central to this. To this end, we will next consider a novel laboratory approach to the investigation of bacterial MICs of sentinel bacteria isolated from the bulk tank of dairy farms.

Chapter 4; An exploratory study to define a minimum inhibitory concentration of mixed culture of sentinel bacteria

4.1 Introduction

In Chapter 1, the necessity for the monitoring of antimicrobial susceptibilities via surveillance programmes was identified as a cornerstone in tackling the challenge posed by AMR. Of the many laboratory methods available for AST of bacteria (Jenkins and Schuetz, 2012), the determination of the MIC via microdilution methods has been described as the 'gold-standard'. However, turnaround of results can take a number of days (Schumacher et al., 2018). Therefore, a process for the generation of MIC data which has been simplified to require less processing time may prove to be of value in the continued monitoring of AMR. In the work carried out in Chapters 2 and 3, bacterial culture of bulk tank milk samples was used to isolate a minimum of six and three isolates of *E. coli* and *Enterococcus* spp. respectively. This was to ensure representativeness of the whole bulk milk sample. To this end, a 'mixed' approach was investigated, whereby a single MIC value representing multiple isolates in a mixed culture representing the whole sample could be obtained.

To consider this, bacterial isolates used in the work constituting Chapter 2 were recovered from frozen storage. The influence of storage conditions on the growth of bacteria recovered from biological sources, which may inadvertently lead to a change in MIC profiles of bacterial isolates, has been acknowledged and investigated (Poulsen et al., 2021). The work presented in this Chapter was carried out approximately 14 months after the microbiology undertaken for the work in Chapter 2. Therefore, initial work to repeat AST for selected bacterial isolates was carried out, followed by investigation of an approach to develop a 'mixed MIC' method. It was hypothesised that, in the presence of a selection pressure (the antimicrobial), the most resistant bacterial isolate in a mixed culture would represent the highest MIC observed across the range of tested concentrations for each antimicrobial tested.

4.2 Materials and methods

4.2.1 Repeat testing and exploratory investigation of 'mixed' method

To test for potential changes in bacterial MIC profiles following frozen storage, a convenience sample of bacterial isolates were selected from the dataset generated in Chapter 2 from the initial bacterial culture and AST of bulk tank milk samples (2.2.5.3). From the methods in Chapter 2, a minimum of six isolates of each bacterial species were selected for AST following bulk tank milk culture for each sampling event. As part of the investigations carried out in this Chapter utilising bacterial isolates from Chapter 2, isolates retrieved from frozen storage were selected as groups of six isolates, as to replicate sample sizes used previously. Farms where bulk tank samples originated were randomly selected and not deemed to be important as part of this convenience sample. The final chosen sample consisted of 72 isolates (*E. coli*; n= 18, *E. faecalis*; n= 41, *E. faecium*; n= 10, *E. durans*; n= 3) selected from twelve farms across four sampling events (August 2017, August 2018, August 2019 and November 2019). All isolates to be recovered had been stored at -80°C using the Protect Microorganism Preservation System (Technical Service Consultants Ltd, Heywood, UK) and suspended on glycerol beads. Isolates were pure plated onto fresh Columbia (5% sheep blood) agar from glycerol beads and incubated for 18-24 hours at 37°C. AST was carried out using Micronaut-S Mastitis 3 microdilution plates (plate configuration provided in Chapter 2, (Table 2.3) according to the procedure previously described (Chapter 2, 2.2.5.2).

A 'mixed MIC' approach, where a single MIC profile is obtained from multiple bacterial isolates, was first considered during this initial stage of AST. From the Columbia (5% sheep blood) agar plates on which the six bacterial isolates selected for testing per farm were cultured, a single cotton swab was used to collect bacteria from each of all the six individual colonies. The cotton swab was placed in a vial of 5ml of demineralised water and rotated against the side of the vial to create a bacterial suspension. For AST, a 0.5 MacFarland turbidity standard was required, which is equal to an optical density of of 1.5 x $10^{\wedge 8}$ colony forming units (CFU) per ml, as determined

by the Sensititre Nephelometer (Thermo Scientific, Massachusetts, USA). Once this was achieved, the standard process for carrying out AST according to procedures outlined when using the Micronaut-S Mastitis 3 microdilution plates was conducted. Figure 4.1 provides a diagrammatic outline of the procedure carried out in the determination of a 'mixed' MIC. Individual bacterial isolate testing and 'mixed' MIC results were collated in an Excel spreadsheet (Microsoft Excel, Microsoft Corporation, 2016).

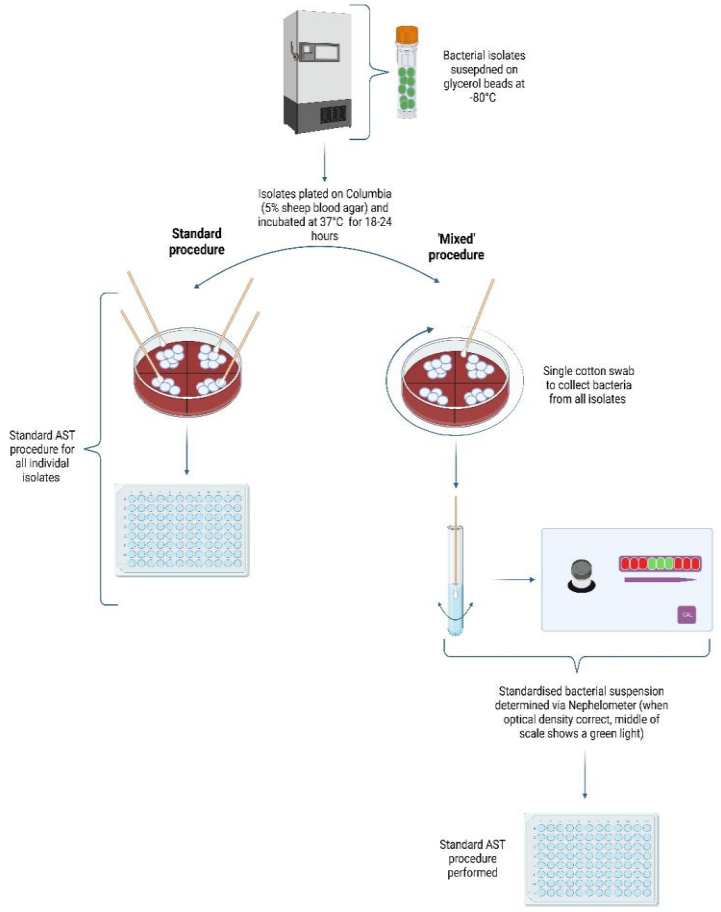

Figure 4.1. A diagrammatic overview of the steps involved for determining a 'mixed' MIC in comparison to standard antimicrobial susceptibility testing of individual bacterial isolates. (Created with BioRender.com)

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4.2.2 Further exploratory investigations of 'mixed' method

Following initial AST of isolates from frozen storage, both individually and through the investigation of a 'mixed' method, a ~~separate~~ second subset of bacterial isolates were selected from the dataset generated in Chapter 2. Isolates to be included, and subsequently arranged in groups of six as to replicate previous testing groups, were selected on the basis of variation between MIC profiles. ~~,~~ In doing so, ~~whereby~~ isolates with generally higher MICs could be compared with those exhibiting lower MICs in the investigation of the 'mixed' MIC. ~~–~~Where isolates had previously been selected according to farm and corresponding sampling period, this was not considered here. ~~Instead, individual isolates to be tested as a group of six were selected across various farms of origin and sampling periods.~~ A convenience sample of 18 isolates were chosen (*E. coli*; n= 6, *E.* faecalis; n= 6, *E. faecium*; n= 6). These isolates were organised into groups of six, as to represent the methodology carried out in Chapter 2 and were referred to as 'test groups'. For ease of retrieval from frozen storage, each individual isolate was plated from glycerol beads twice. Thereby, six test groups were formed, whereby *E. coli* constituted two test groups and a further two test groups for *E. faecalis* and *E. faecium* respectively. Due to the smaller proportion of *E. durans* present within the dataset isolates were sourced from, none were included at this point. AST was carried out both at the individual isolate level and via the 'mixed' MIC approach as outlined in the previous stage of isolate testing. All results generated at this stage were collated in an Excel spreadsheet (Microsoft Excel, Microsoft Corporation, 2016).

A second subset of bacterial isolates was formed as part of a final stage in the exploratory investigations of 'mixed' MICs, which aimed to introduce variability into the test groups. Groups of isolates be included for AST for *Enterococcus* spp. consisted of a mix of the three species (*E. faecalis*, *E. faecium* and *E. durans*). The final isolates included in this subset sample was formed as follows; *E. coli*; n=12, *E. faecalis*; n=8, *E. faecium*; n=6, *E. durans*; n=4, and formed two test groups of *E. coli* and four for *Enterococcus* spp. All isolates were plated once, rather than twice as with the previous sample subset. AST was carried out both at the individual isolate level and

via the 'mixed' MIC approach as in the previous stage of isolate testing. The 'mixed' MIC approach was repeated five times to investigate agreement between 'mixed' measurements. An overview of the methodology in forming the subsets of isolates is provided in Figure 4.2. All results generated from this final stage were collated in an Excel spreadsheet (Microsoft Excel, Microsoft Corporation, 2016).

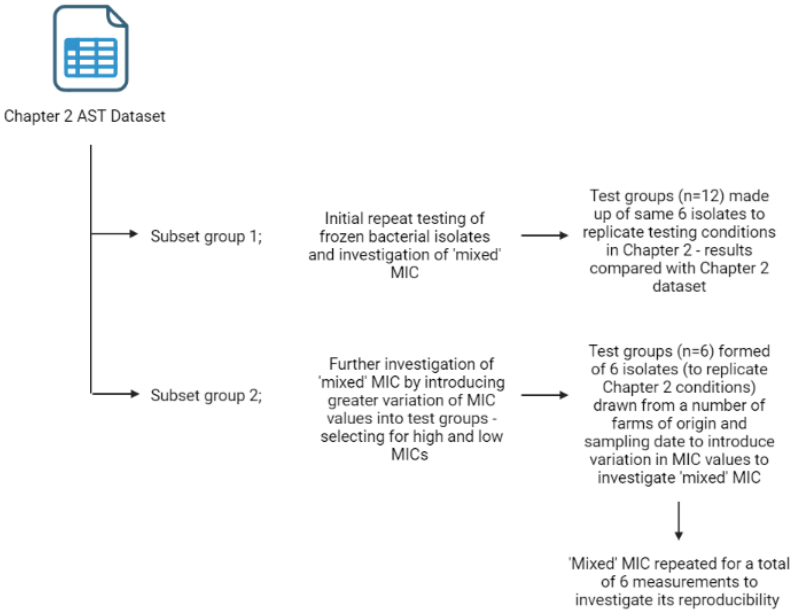


Figure 4.2. An overview of the creation of the two subsets of bacterial isolates for investigation of change in MIC after freezing and subsequent 'mixed' MIC investigation. (Created with BioRender.com)

Commented [DM9]: Flow diagram for isolate subsamples forming methodology of this chapter

4.2.3 Data analysis

Following AST of each subset of bacterial isolates, all data were examined to identify concordance between: (i) MICs of isolates following an extended period of frozen storage and MIC values generated during initial repeat testing and (ii) MICs of individual isolates and the respective 'mixed' MIC value.

4.2.3.1 Minimum inhibitory concentration profiles following frozen storage

Analysis of concordance between MICs for each antimicrobial tested across all *Enterococcus* spp. and *E. coli* isolates generated previously and those following frozen storage was measured via calculation of percentage agreement using the *irr* package in R (Gamer et al., 2019). Percentage agreement (PA) was calculated according to the following formula;

$$PA = \frac{No.of\ measurements\ with\ same\ MIC\ value}{No.of\ total\ measurements\ observed}\ x\ 100$$

Two measures of concordance via percentage agreement were made. First, a measure of absolute concordance was made, where agreement was defined as the MICs in both instances being exactly the same. The second measure took into account the inherent error of the MIC to provide results as ± one microdilution, where results were examined visually to deduce percentage agreement.

4.2.3.2 Mixed method

Comparisons of MIC profiles generated for the 'mixed' approach used those of individual isolates as a reference. Results were arranged in tables according to each subsample test group. For analysis, the MIC value for each antimicrobial of the 'mixed' sample was deemed to be representative when this value corresponded with the highest MIC value seen across all individual isolates in the test group. The existence of ± one microdilution was also taken into account when considering the 'mixed' MIC value in relation to the reference isolates.

4.3 Results

4.3.1 Comparison of MIC following period of frozen storage

Measures of concordance between MICs of frozen isolates and MICs measured during repeat testing for selected *Enterococcus* spp. and *E. coli* isolates are provided in Table 4.1**.** Higher levels of agreement between test groups were seen for *E. coli* isolates, when compared with *Enterococcus* spp. isolates, where five (45.5%) of antimicrobials tested had 100% agreement, compared to only one (9%) for *Enterococcus* spp. The lowest level of percentage agreement for *E. coli* isolates was 81.2% for amoxicillin/clavulanic acid and kanamycin/cefalexin.

Table 4.1~~4.1~~**.** An outline of concordance measured by exact percentage agreement of MICs generated for isolates in Chapter 2 and after a period of frozen storage for each antimicrobial for *Enterococcus* spp. (n=54) and *E. coli* (n=18) isolates.

Antimicrobial	*Enterococcus* spp.	*E. coli*
Amoxicillin/Clavulanic acid	100	81.2
Ampicillin	98.1	87.5
Cefazolin	72.2	87.5
Cefoperazon	87	93.8
Cefquinome	57.4	93.8
Erythromycin	63	100
Kanamycin/Cefalexin	59.3	81.2
Marbofloxacin	64.8	100
Oxacillin	88.9	100
Penicillin G	57.4	100
Pirlimycin	70.4	100

When concordance between test groups was considered further to account for MICs featuring ± one microdilution, MICs were almost in perfect agreement for both *Enterococcus* spp. and *E. coli*. For *Enterococcus* spp., all antimicrobials tested had 100% agreement apart from cefquinome and oxacillin, where two isolates featured MICs which had a discrepancy of ≥ 2 microdilutions which gave a final agreement for both antimicrobials of 96.4%. For *E. coli*, 7 of the 11 antimicrobials tested had 100% agreement. Agreement for ampicillin was at 87.5% with two isolates having a discrepancy of ≥ 2 microdilutions. All three cephalosporins (cefazolin, cefoperazon and cefquinome) each had one isolate featuring a discrepancy of ≥ 2 microdilutions, with a final agreement of 93.8% each.

4.3.2 'Mixed' minimum inhibitory concentrations

Investigation of the 'mixed' MIC approach as a representative measure of individual isolates showed overall high levels of agreement between MICs for antimicrobials tested as individual isolates as a reference and 'mixed' MIC value.

Table 4.2 and Table 4.3 provide an overview of data generated for the 12 test groups where a 'mixed' method was tested from those isolates initially used to make comparisons following a period of frozen storage for *E.coli* and *Enterococcus* spp. respectively. For the *E. coli* test groups (n=3), two had a 'mixed' MIC profile which was in complete agreement with the reference isolate MICs, while one only differed by ± one microdilution for amoxicillin/clavulanic acid (Table 4.2, Mixed 2).

For *Enterococcus* spp. test groups considered at this first instance of 'mixed' MIC investigation (n= 9), one had a 'mixed' MIC profile which was representative of the highest MIC of individual isolates across all antimicrobials (Table 4.3, mixed measure 6). The remainder of test groups (n=8) featured at least one MIC in the mixed profile which was ± one microdilution from the highest MIC for antimicrobials tested against individual isolates. Of these, two test groups featured a single MIC which was ≥ 2 microdilutions higher or lower than the highest MIC amongst individual reference isolates (Table 4.3, mixed measures 1 and 2).

Table 4.2~~4.2~~. Overview of MIC profiles generated from *E. coli* isolates (n=18) which underwent AST in the investigation of the effect of frozen storage and the subsequent determination of a mixed MIC. Green shading for the mixed measure indicates the highest MIC measured from individual reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC for an individual reference isolate.

Isolate ID	Antimicrobials										
	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J13	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J14	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J15	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J16	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J17	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J18	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J79	<=4	>16	<=4	<=2	<=1	>4	32	<=0.25	>4	>8	>4
J80	8	>16	<=4	<=2	<=1	>4	32	<=0.25	>4	>8	>4
J82	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J84	8	>16	<=4	<=2	<=1	>4	32	<=0.25	>4	>8	>4
J85	8	<=4	<=4	<=2	<=1	>4	8	<=0.25	>4	>8	>4
J86	8	<=4	<=4	<=2	<=1	>4	32	<=0.25	>4	>8	>4
Mixed 2	<=4	>16	<=4	<=2	<=1	>4	32	<=0.25	>4	>8	>4
J1533	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1534	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1535	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1536	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1537	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1538	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 3	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4

Table 4.3~~4.3~~. Overview of MIC profiles generated from *Enterococcus spp.* isolates (n=54) which underwent AST in the investigation of the effect of frozen storage and the subsequent determination of a mixed MIC. Green shading for the mixed measure indicates the highest MIC measured amongst reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC amongst reference isolates. Red shading indicates a measure of ≥ 2 microdilutions higher or lower than the highest MIC for reference isolates.

	Antimicrobials										
Isolate ID	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J121	<=4	<=4	32	>16	4	1	16	1	>4	1	4
J122	<=4	<=4	32	>16	8	1	16	1	>4	4	4
J123	<=4	<=4	32	>16	4	0.5	16	1	>4	2	4
J124	<=4	<=4	32	>16	4	0.5	16	1	>4	2	4
J129	<=4	<=4	32	>16	4	1	16	1	>4	2	4
J130	<=4	<=4	32	>16	4	1	16	1	>4	2	4
Mixed 1	<=4	<=4	32	>16	4	1	>32	1	>4	2	4
J125	<=4	<=4	32	>16	4	0.5	32	1	>4	2	4
J126	<=4	<=4	16	16	4	2	32	1	>4	2	4
J127	<=4	<=4	16	16	4	1	32	1	>4	2	4
J128	<=4	<=4	32	>16	4	0.5	32	1	>4	4	2
J131	<=4	<=4	32	>16	4	2	>32	1	>4	2	4
J132	<=4	<=4	32	>16	4	1	32	1	>4	2	4
Mixed 2	<=4	<=4	32	>16	4	4	32	1	4	1	4
J156	<=4	<=4	16	16	4	0.5	32	0.5	>4	2	2
J157	<=4	<=4	32	8	8	2	32	1	>4	1	4
J158	<=4	<=4	32	8	4	1	32	1	>4	1	4
J160	<=4	<=4	32	8	4	2	>32	1	>4	1	4
J161	<=4	<=4	32	>16	8	>4	>32	1	>4	2	>4
J162	<=4	<=4	16	16	4	0.5	32	0.5	>4	2	2
Mixed 3	<=4	<=4	32	>16	4	>4	>32	1	>4	2	>4
J163	<=4	<=4	>32	>16	>8	0.25	>32	1	>4	4	>4
J164	<=4	<=4	16	16	2	0.5	8	1	>4	1	1
J165	<=4	<=4	8	8	<=1	0.5	16	0.5	>4	1	2
J166	<=4	<=4	>32	>16	>8	0.5	32	2	>4	4	>4
J167	<=4	<=4	32	>16	4	0.5	32	1	>4	1	2
J168	<=4	<=4	>32	>16	>8	2	32	>2	>4	4	<=1
Mixed 4	<=4	<=4	>32	>16	>8	1	>32	2	>4	4	>4

Table 4.3 Continued											
J193	<=4	<=4	32	>16	4	1	16	1	>4	2	4
J194	<=4	<=4	32	>16	4	1	16	1	>4	2	4
J195	<=4	<=4	32	>16	4	1	16	1	>4	2	2
J196	<=4	<=4	32	>16	8	2	32	0.5	>4	4	4
J197	<=4	<=4	32	>16	8	1	32	0.5	>4	4	4
J198	<=4	<=4	32	>16	4	1	32	0.5	>4	2	2
Mixed 5	<=4	<=4	32	>16	8	2	32	1	>4	8	4
J233	<=4	<=4	32	>16	4	>4	>32	1	>4	2	>4
J234	<=4	<=4	16	16	4	2	32	1	>4	1	4
J235	<=4	<=4	32	>16	4	0.5	32	1	>4	2	4
J236	<=4	<=4	>32	>16	>8	4	32	>2	>4	4	>4
J237	<=4	<=4	>32	>16	>8	>4	32	>2	>4	4	>4
J238	<=4	<=4	>32	>16	>8	4	32	>2	>4	4	>4
Mixed 6	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	4	>4
J711	<=4	<=4	>32	>16	>8	0.5	>32	>2	>4	4	>4
J712	<=4	<=4	>32	16	>8	<=0.125	8	0.5	>4	2	<=1
J713	<=4	<=4	>32	16	8	0.5	32	1	<4	2	4
J714	<=4	<=4	16	16	2	1	32	1	<4	2	4
J715	<=4	<=4	16	16	<=1	0.5	32	0.5	4	1	2
J716	<=4	<=4	32	16	4	1	32	0.5	<4	2	2
Mixed 7	<=4	<=4	>32	>16	>8	1	>32	2	>4	4	>4
J916	<=4	<=4	>32	>16	2	2	16	>2	>4	4	<=1
J917	<=4	<=4	>32	>16	4	1	32	2	>4	2	<=1
J918	<=4	<=4	32	>16	4	0.5	>32	1	>4	4	4
J919	<=4	<=4	>32	>16	>8	1	>32	1	>4	4	4
J920	<=4	<=4	>32	>16	>8	0.5	8	<=0.25	>4	2	<=1
J921	<=4	<=4	>32	>16	>8	<=0.125	32	<=0.25	>4	2	<=1
Mixed 8	<=4	<=4	>32	>16	>8	2	32	2	>4	4	>4
J1423	<=4	<=4	32	>16	4	0.5	32	1	>4	2	4
J1424	<=4	<=4	32	>16	4	0.5	32	0.5	>4	2	<=1
J1425	<=4	<=4	16	>16	4	0.5	>32	0.5	>4	2	<=1
J1426	<=4	<=4	32	>16	4	1	32	0.5	>4	2	<=1
J1427	<=4	<=4	<=4	<=2	<=1	1	16	2	<=1	0.25	>4
J1428	<=4	<=4	16	4	<=1	1	16	2	<=1	0.25	>4
Mixed 9	<=4	<=4	32	>16	4	1	32	2	>4	2	>4

Table 4.4 and Table 4.5 represent the outcomes of the determination of mixed MIC profiles for *E. coli* and *Enterococcus* spp. isolates respectively when isolated were tested individually and via the mixed MIC approach when isolates were selected for variability in the second stage of exploratory investigations. For the *E. coli* test groups (n=2), one featured complete agreement between the mixed profile and reference isolates while the other featured only one antimicrobial which differed by ± one microdilution. For the *Enterococcus* spp. test groups (n=4) all four mixed profiles featured one MIC which was ± one microdilution when compared to the individually tested isolates as a reference.

Table 4.4~~4.4~~. Overview of MIC profiles generated for *E. coli* test groups (n=2) where individual isolates to be used as a reference were selected based on variability in their own MIC profiles and subsequent determination of mixed MIC. Green shading for the mixed measure indicates the highest MIC measured amongst reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC amongst reference isolates.

	Antimicrobials										
Isolate ID	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J1520	<=4	<=4	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
J1528	<=4	8	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
1557	32	>16	>32	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1	32	>16	>32	<=2	1	>4	<=4	<=0.25	>4	>8	>4
J478	32	>16	<=4	<=2	8	>4	8	<=0.25	>4	>8	>4
J481	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1461	<=4	<=4	<=4	<=2	<=1	>4	>32	<=0.25	>4	>8	>4
Mixed 2	32	>16	<=4	<=2	8	>4	>32	<=0.25	>4	>8	>4

Table 4.5~~4.5~~. Overview of MIC profiles generated for *Enterococcus* spp. test groups (n=4) where individual isolates to be used as a reference were selected based on variability in their own MIC profiles. Green shading for the mixed measure indicates the highest MIC measured amongst reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC amongst reference isolates.

	Antimicrobials										
Isolate ID	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J164	<=4	<=4	16	16	2	0.5	16	1	>4	2	<=1
J168	<=4	<=4	>32	>16	>8	2	>32	>2	>4	4	<=1
J969	<=4	<=4	32	8	8	2	32	1	>4	1	4
Mixed 1	<=4	<=4	>32	>16	>8	2	>32	2	>4	4	4
J200	<=4	<=4	32	>16	>8	0.5	32	1	>4	2	4
J369	<=4	<=4	32	8	4	2	32	1	>4	1	4
J370	<=4	<=4	16	16	4	1	32	1	>4	2	4
Mixed 2	<=4	<=4	32	>16	8	2	32	1	>4	2	4
J214	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	>8	>4
J989	<=4	<=4	32	4	<=1	1	16	>2	4	<=0.125	>4
J991	<=4	<=4	32	4	<=4	1	16	>2	4	<=0.125	>4
Mixed 3	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	8	>4
J1169	<=4	<=4	8	4	<=1	1	16	2	2	<=0.125	>4
J1174	<=4	<=4	>32	>16	>8	4	>32	>2	>4	4	>4
J1179	<=4	<=4	>32	>16	>8	2	>32	2	>4	4	>4
Mixed 4	<=4	<=4	>32	>16	>8	4	>32	2	>4	4	>4

Table 4.6 and Table 4.7 represent the outcomes of the determination of repeated mixed MIC profiles for *E. coli* and *Enterococcus* spp. isolates as part of the final stage of exploratory investigations. For the *E. coli* test groups (n=2), neither featured complete agreement between repeated measures of the mixed MIC approach with individual isolates as references, but agreement was good amongst most antimicrobials. For mixed measures 1.1-1.6, (Table 4.6) agreement between all antimicrobials apart from cefazolin was found, where four of the repeat measures were ± one microdilution away from the highest reference MIC, while the remaining two measures were two microdilutions less than the highest reference MIC. For the other test group (mixed measures 2.1-2.6), complete agreement was found between all antimicrobials between repeated mixed measures and the highest reference MICs apart from cefquinome. Here five out six of the repeated measures were one microdilution lower than the reference.

For the *Enterococcus* spp. test groups (n=4) as outlined in Table 4.7, complete agreement between repeated mixed MIC measures as well as with reference isolates was good. However, discrepancies of ± one microdilutions were found across test groups, but no differences of ≥ 2 microdilutions were found.

Table 4.6~~4.6~~. Overview of MIC profiles generated for *E. coli* test groups (n=2) where mixed MIC measures were repeated. Green shading for the mixed measure indicates the highest MIC measured amongst reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC amongst reference isolates. Red shading indicates a measure of ≥ 2 microdilutions higher or lower than the highest MIC for reference isolates.

	Antimicrobials										
Isolate ID	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J305	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J306	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J308	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J309	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J488	32	>16	32	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J1537	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.1	32	>16	16	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.2	32	>16	16	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.3	32	>16	16	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.4	32	>16	16	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.5	32	>16	8	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
Mixed 1.6	32	>16	8	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J470	<=4	<=4	<=4	<=2	<=1	>4	<=4	<=0.25	>4	>8	>4
J473	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
J475	32	>16	<=4	<=2	4	>4	<=4	<=0.25	>4	>8	>4
J476	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
J477	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
J478	32	>16	<=4	<=2	4	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.1	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.2	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.3	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.4	32	>16	<=4	<=2	4	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.5	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4
Mixed 2.6	32	>16	<=4	<=2	2	>4	<=4	<=0.25	>4	>8	>4

Table 4.7~~4.7~~. Overview of MIC profiles generated for *Enterococcus* spp. test groups (n=4) where mixed MIC measures were repeated. Green shading for the mixed measure indicates the highest MIC measured amongst reference isolates is represented in the MIC profile. Orange shading indicates a measure of ± one microdilution away from the highest MIC amongst reference isolates.

	Antimicrobials										
Isolate ID	Amoxicillin Clavulanic acid	Ampicillin	Cefazolin	Cefoperazon	Cefquinome	Erythromycin	Kanamycin/ Cefalexin	Marbofloxacin	Oxacillin	Penicillin G	Pirlimycin
J200	<=4	<=4	32	>16	>8	0.5	32	1	>4	2	2
J369	<=4	<=4	8	8	4	2	32	1	>4	1	4
J744	<=4	<=4	>32	16	4	>4	>32	2	>4	1	>4
J788	<=4	<=4	>32	>16	>8	<=0.125	8	0.5	>4	2	<=1
J789	<=4	<=4	>32	>16	>8	<=0.125	8	0.5	>4	2	<=1
J792	<=4	<=4	32	>16	4	1	16	1	>4	2	4
Mixed 1.1	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
Mixed 1.2	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
Mixed 1.3	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
Mixed 1.4	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
Mixed 1.5	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
Mixed 1.6	<=4	<=4	>32	>16	>8	>4	>32	2	>4	1	>4
J396	<=4	<=4	16	16	<=1	0.5	32	1	2	1	2
J397	<=4	<=4	32	16	4	0.5	32	1	>4	2	2
J398	<=4	<=4	32	>16	8	<=0.125	32	1	>4	2	4
J785	<=4	<=4	>32	16	>8	<=0.125	8	0.5	>4	2	<=1
J989	<=4	<=4	<=4	<=2	<=1	1	16	2	<=1	<=0.125	>4
J992	<=4	<=4	<=4	<=2	<=1	1	32	2	<=1	<=0.125	>4
Mixed 2.1	<=4	<=4	>32	>16	>8	1	32	>2	>4	2	>4
Mixed 2.2	<=4	<=4	>32	>16	>8	1	32	2	>4	2	>4
Mixed 2.3	<=4	<=4	>32	>16	>8	1	32	2	>4	2	>4
Mixed 2.4	<=4	<=4	>32	>16	>8	1	32	2	>4	2	>4
Mixed 2.5	<=4	<=4	>32	>16	>8	1	32	2	>4	2	>4
Mixed 2.6	<=4	<=4	>32	>16	>8	1	32	2	>4	2	>4
J833	<=4	<=4	>32	8	<=1	2	>32	>2	>4	4	<=1
J837	<=4	<=4	>32	>16	4	>4	>32	1	>4	2	>4
J838	<=4	<=4	32	>16	4	0.5	>32	1	>4	2	2
J854	<=4	<=4	>32	>16	>8	1	32	2	>4	4	<=1
J862	<=4	<=4	32	16	4	0.5	>32	<=0.25	>4	2	<=1
J864	<=4	<=4	>32	8	>8	<=0.125	16	<=0.25	>4	2	>4
Mixed 3.1	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	4	>4
Mixed 3.2	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	8	>4
Mixed 3.3	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	4	>4

Table 4.7 Continued											
Mixed 3.4	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	8	>4
Mixed 3.5	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	4	>4
Mixed 3.6	<=4	<=4	>32	>16	>8	>4	>32	>2	>4	8	>4
J1346	<=4	<=4	32	>16	4	1	32	1	>4	2	2
J1347	<=4	<=4	32	>16	4	1	32	1	>4	2	2
J1350	<=4	<=4	>32	>16	>8	<=0.125	<=4	0.5	>4	1	<=1
J1355	<=4	<=4	>32	>16	>8	<=0.125	<=4	0.5	>4	1	<=1
J1358	<=4	<=4	>32	>16	>8	2	>32	>2	>4	4	<=1
J1360	<=4	<=4	>32	>16	>8	4	>32	>2	>4	4	<=1
Mixed 4.1	<=4	<=4	>32	>16	>8	4	>32	>2	>4	4	2
Mixed 4.2	<=4	<=4	>32	>16	>8	4	>32	>2	>4	4	2
Mixed 4.3	<=4	<=4	>32	>16	>8	4	>32	>2	>4	4	2
Mixed 4.4	<=4	<=4	>32	>16	4	2	>32	>2	>4	4	2
Mixed 4.5	<=4	<=4	>32	>16	4	2	>32	>2	>4	4	2
Mixed 4.6	<=4	<=4	32	>16	>8	4	>32	>2	>4	4	2

4.4 Discussion

The implementation of surveillance procedures involving the monitoring of antimicrobial susceptibilities allows for informed decision making, including directing new policy approaches and informing the direction of future surveillance. Laboratory generated data allows for the determination of bacterial susceptibilities to antimicrobials and emerging trends to be monitored longitudinally in areas of geographic interest (Johnson, 2015).

In Chapter 2, antimicrobial susceptibilities of sentinel bacteria isolated from bulk tank milk samples were considered as individual isolates, with the aim of isolating a minimum of six isolates of *Enterococcus* spp. and *E. coli* to ensure a representative sample for each farm was being achieved. Such an approach, if applied to a large number of samples requiring processing, constitutes a high input in terms of raw materials and labour. Therefore, a more simple laboratory method would be valuable to monitor AMR.

The aim of this work was to investigate measuring MICs based on a 'mixed' isolate approach, whereby a single MIC profile represents the highest MIC for a range of given antimicrobials. This measure has been referred to as a 'mixed' MIC. Such an

approach is not common in published literature concerning the monitoring of antimicrobial susceptibilities, particularly in agriculture or veterinary medicine research. The main source of previous research relating to a 'mixed' MIC approach comes from research in the field of human medicine, particularly concerning infection of cystic fibrosis patients with *Pseudomonas aeruginosa* (Van Horn, 1993; Morlin et al., 1994). AST in order to select appropriate antimicrobial therapy is often complicated by the presence of multiple morphotypes that may display varying susceptibilities. The authors of these studies note that AST was based on isolating individual morphotypes, but acknowledged the potential value of testing a single sample which encapsulated multiple *P. aeruginosa* morphotypes. Both studies found high levels of agreement between the MICs of individually tested isolates and a mixed sample of multiple morphotypes. Morlin et al., (1994) concluded that isolation of individual bacterial samples was a 'gold standard' approach but their 'mixed' approach may be more representative of a *P. aeruginosa* infection of a cystic fibrosis patient's lungs. They also noted that this method had a decreased turn around time in terms of sample processing and was less expensive.

Therefore, an investigation of the value of a 'mixed' MIC method for monitoring AMR in bulk tank milk is warranted. The hypothesis of this investigation was that, in the presence of a selection pressure (the antimicrobial), the most resistant bacterial isolate in a mixed culture would represent the highest MIC observed across the range of tested concentrations for each antimicrobial tested.

Interpretation of the results of this work required concordance between results to be measured. When measuring concordance between test groups, a number of methods can be employed, ranging from calculation of percentage agreement to use of statistical tests such as Cohen's kappa. Cohen's kappa is a measure of agreement between two observations, but also considers the presence of agreement by chance, which percentage agreement does not (Sun, 2011). It has been noted that there can be there can be a high measure of concordance when using percentage agreement, but a low measure when using Cohen's kappa (Dettori and Norvell, 2020). In calculating chance, Cohen's kappa makes assumptions about the dataset and overcorrect for the presence of chance observations. Additionally, difficulties in its

Commented [DM10]: Comments on % agreement vs cohens kappa

interpretation may lead to an acceptance of measures of concordance when there may actually be high levels of concordance in the dataset (McHugh, 2012). Given that the data had to be inspected manually to account for ± one microdilution which is inherently accepted with the MIC measurement, it was believed that use of Cohen's kappa over would have skewed the interpretation of concordance to much lower levels than was reflected in the data. Therefore, percentage agreement was used to interpret concordance between results.

As this work was utilising bacterial isolates which had been previously processed, it was decided that these should be tested again to investigate any potential changes in MIC profiles following frozen storage. When comparing MIC profiles of the original AST and that which was carried out for this work, changes in profiles were observed. When considering the comparison of raw MIC categories, higher levels of concordance were seen for *E. coli* compared to the MIC profiles for *Enterococcus* spp. However, when results were inspected further to consider the effect of ± one microdilution error which is associated with the MIC, the levels of concordance in terms of percentage agreement were very high, with a low of 96.4% and 87.5% agreement for *Enterococcus* spp. and *E. coli* respectively. With regards to the outcomes in terms of agreement between individual isolate MICs and those obtained for the 'mixed' MIC samples, overall high degrees of agreement were found. Although some discrepancies were observed, when taken into account in the context of ± one microdilution error for the MIC measurement, the 'mixed' measure was almost always representative of the highest MICs for individually tested isolates found for each antimicrobial tested. When investigating their 'mixed' MIC approach, Van Horn (1993) and Morlin et al., (1994) found correlations between 'mixed' samples and individual isolates in terms of percentage agreement (including consideration of ± one microdilution) of 96% and 73.5% respectively. These data suggest that a 'mixed' morphotype, or in the case of the work presented in this Chapter, 'mixed' isolate, provide a high degree of concordance, highlighting a convenient yet representative method to establish MIC profiles of bacterial species of interest in an AMR monitoring procedure.

4.5 Study limitations

Isolates which were selected for testing as part of the work outlined in this Chapter were sourced from frozen storage which had originally undergone AST as part of the research outlined in Chapter 2. The overall subsample selected for analysis in this Chapter was smaller. A larger sample size may have allowed for a better consideration of the changes in MICs following frozen storage as well as in the investigation of the 'mixed' MIC method.

Additionally, the uncertainty over the influence of the error of the MIC as a measurement may have limited the interpretation of results presented in this Chapter. An effort to account for the potential impact of this via consideration of raw data for MIC results which were ± one microdilution from reference MIC measures was employed. Therefore, there is some uncertainty as to whether the MIC profiles for each antimicrobial tested was due to actual changes or due to measurement error.

4.6 Conclusions

The outcomes of the work presented in this Chapter demonstrate that, in a mixed isolate culture, an MIC profile which reflects the most resistant bacteria within that culture can be determined across a number of tested antimicrobials. This highlights the potential for a more representative measure of resistance to be determined on dairy farms via bulk tank milk sampling. In addition, it represents a more convenient method of AST when considering multiple bacterial isolates cultured from a single sample, where less processing and time is required. As a result, these findings signify the potential for further investigation of the development of a laboratory AST method which is both representative of a whole bulk tank milk sample and convenient, whilst accurately reflecting antimicrobial susceptibilities. To this end, we consider the development of a novel approach to address this which is presented in Chapter 5 of this thesis.

Chapter 5; investigating the suitability of a novel laboratory method for the monitoring of antimicrobial susceptibilities

5.1 Introduction

There are a number of methods available for the AST of bacteria, such as agar dilution, agar disc diffusion, broth micro/macro dilution (Jenkins and Schuetz, 2012) and the use of genomics allows for the identification of AMR related genes (Hendriksen et al., 2019).

A method which is low cost and requires less materials and labour, however, would prove to be of value in antimicrobial monitoring programmes. In Chapter 4, the representativeness of a single MIC value, which reflects the highest MIC value of each antimicrobial across multiple isolates was investigated. The conclusions drawn from that study informed the work carried out in this Chapter. From this, one MIC value was assumed to represent antimicrobial susceptibilities of a select bacterial population derived from bulk tank milk samples. Bulk tank milk samples were sourced from local farms for ease of sample collection. Where prior AST presented in this thesis consisted of culturing and selecting individual bacterial isolates from selective agar and subsequent culture on non-selective agar, in this study, nutrient broths were used to enrich raw bulk tank milk samples. Antimicrobial susceptibilities were then determined directly from this broth following a period of incubation, without any subculture on agar. Such a process has not, to the author's knowledge, been investigated or recommended for any routine AST. An attempt to validate this approach was undertaken, by which comparisons with MICs generated from bacteria cultured on agar were made.

Two overarching aims constituted the work outlined here: (i) the viability of ~~antimicrobial susceptibility testing~~AST direct from broth culture and (ii) whether regular sampling of bulk tank milk samples through a monitoring programme could detect changes in bacterial MICs over time. Initial investigations regarding use of nutrient broths to enrich bulk tank milk samples were made in a pilot study, which informed how later work would be carried out in order to investigate these aims.

5.2 Materials and methods

5.2.1 Investigating a potential sample processing methodology

A pilot study was undertaken to investigate a potential methodology to obtain MIC profiles of sentinel bacteria isolated from bulk tank milk samples. This approach was based on the use of selective nutrient broths, rather than selective agars, to enrich milk samples and perform AST directly from the nutrient broth.

5.2.1.1 Pilot sampling

Pilot milk samples were taken from a convenience sample of five farms local to the laboratory with commercial and/or personal contacts for ease of sample collection. Fresh, chilled, bulk tank milk samples of approximately 100ml were collected by members of laboratory staff by sampling from the bottom outlet of the tanks before a morning milk collection. Samples were received at the laboratory and processed on the same day.

5.2.1.2 Sample processing

Nutrient broths identified for use based on their selective enrichment for *E. coli* and *Enterococcus spp.* were Enterobacteriaceae Enrichment broth (EE) and Azide Dextrose Broth Rothe (AZ) (Oxoid, Basingstoke, UK) respectively. For EE broth, 43.5g of dehydrated culture media was dissolved in 1 litre of distilled water, mixed and transferred in 100ml portions into 250ml flasks. The medium was heated at 100°C using free flowing steam for 30 minutes then cooled rapidly in cold running water. Two concentrations of AZ broth were prepared; single and double strength. For single strength AZ broth, 35.6g of dehydrated culture media was added to one litre of distilled water, with 71.2g (double strength) being added for double strength AZ broth. The medium was heated gently to dissolve and dispensed into 500ml borosilicate glass Schott bottles and sterilised by autoclaving at 121°C for 15 minutes.

Once prepared, each broth type was dispensed in 225ml portions into 250ml borosilicate glass Schott bottles. Nutrient broths were prepared the morning bulk tank samples were received, with any extra being placed in cold storage until required.

When ready for processing, bottles of nutrient broths were labelled according to farm sample. For each farm, 25ml of milk were added to the 225ml portions of EE and both single and double strength AZ broths and incubated at 44°C for 48 hours, but to be checked at 24 hours of incubation.

From the portion of milk remaining, aliquots were taken for direct plating onto agar media to examine the microbiology of the raw milk samples. Aliquots of 10µl were streaked onto non-selective and selective agars: (i) Columbia (5% sheep blood) agar, (non-selective); (ii) Tryptone Bile X-Glucuronide (TBX), (selective for *E. coli*); (iii) Slanetz and Bartley (SB), (selective for *Enterococcus* spp.) and (iv) Edward's medium, (selective for *Streptococcus*). Additionally, aliquots of 100µl were spread on TBX, SB and MacConkey agar (selective for coliforms). Columbia (5% sheep blood), Edwards and MacConkey agars were incubated at 37°C for 72 hours with TBX being incubated at 44°C for 48 hours and SB at 35°C for 4 hours then moved to 44°C for 44 hours (48 hours total).

At 24 hours of incubation, the enrichment broths were checked and taken out of incubation to be plated onto agar to examine how the microbiology of the raw milk samples were being enriched by selective broths. Aliquots of 10µl from each broth were streaked onto Columbia (5% sheep blood), TBX (from EE broth only) and SB (both strengths of AZ broth only). This was repeated with spread plates of 100µl for each broth. TBX and SB plates were incubated at 44°C for 48 hours with the Columbia (5% sheep blood) plates being incubated at 37°C for 72 hours. Enrichment broths were returned to incubation for another 24 hours, after which the same plating process was repeated.

Once incubation for all agar plates was complete, bacterial colonies were identified by MALDI-TOF MS using Biotyper 3.1 (Bruker Daltonics, Coventry, UK). Bacterial

colonies to be identified were selected based on varying morphologies across samples from all farms. All results were collated for later reference.

5.2.1.3 Broth processing

Following 48 hours of incubation, a process for determining the (MIC) of the broth-enriched bacterial population was investigated. According to the Thermofisher Sensititre procedure for AST (Thermo Scientific, Massachusetts, USA), a standard bacterial suspension in demineralised water equalling a 0.5 MacFarland turbidity standard with an optical density of bacterial suspension of 1.5 x $10^{\wedge 8}$ CFU/ml is required. A bacterial suspension of the desired optical density was obtained using a Sensititre Nephelometer (Thermo Scientific, Massachusetts, USA). A representation of this is provided in Figure 5.1. Where a suspension closely matches the MacFarland standard, the middle box of the scale will illuminate green. The boxes either side will illuminate green when the suspension is an acceptable optical density away from the standard. When the red boxes to left and right of the scale illuminate red, this is an indication the suspension is of a much lower or a much higher density respectively.

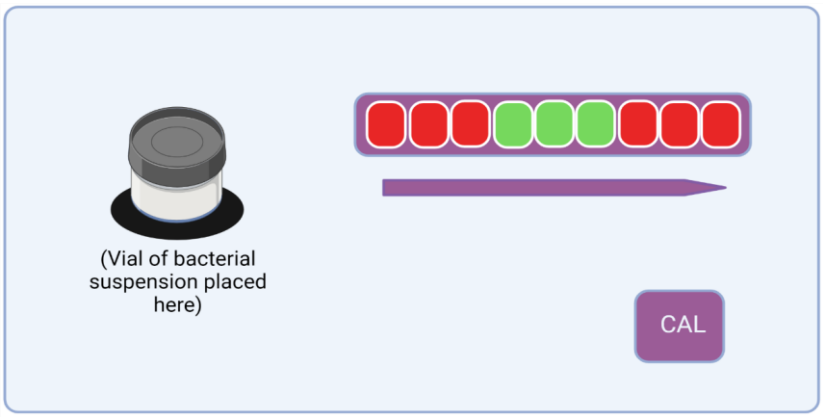


Figure 5.1. A representation of the Sensititre Nephelometer to measure optical density of bacterial suspensions. Red indicates an unfavourable optical density, while green represents a favourable optical density. (Created with BioRender.com)

Initially, a 100µl aliquot of EE broth from Farm A was added to a 5ml vial of demineralised water, giving an optical density which was too high (at the far right hand of the scale). This was then repeated with descending volumes of broth until the optical density was determined to be 'just right' (where the middle green box was illuminated). This process was repeated with all remaining broths across the five sample farms to achieve standardised inoculum, using an initial 25µl aliquots as a baseline against which the desired optical density of inoculum could be identified. AST was carried out using the Micronaut-S Mastitis 3 microdilution plates. Following the inoculation of broth into demineralised water, an aliquot of 50µl of EE and 100µl of AZ broths (single and double strengths) bacterial suspensions was added to 11.5ml vials of Mueller-Hinton broth, as per recommended procedure for Gram-positive and Gram-negative bacteria.

Vial lids were replaced with single use Sensititre dosing heads and placed in the Sensititre Automated Inoculation Delivery System. Following inoculation, an aliquot of inoculant was taken from one of the positive control wells with a 1µl loop and plated onto Columbia (5% sheep blood) agar as a test for inoculant purity, which was incubated for 18-24 hours at 37°C. An adhesive cover was placed over the plate and incubated at 35°C for 18-20 hours. Following the incubation period, purity plates were checked and microdilution plates placed in a Sensititre Vizion and read manually via the SWIN™ software (Thermo Scientific, Massachusetts, USA). Plate images were saved as JPEG files for future reference, with MIC results being entered into an Excel spreadsheet for later analysis.

Purity plates from EE broth samples lacked visible bacterial growth, so were placed back into the incubator for another 4 hours. Microdilution plates either lacked growth or displayed patterns of growth which were difficult to interpret. Purity plates for Azide Dextrose broths (single and double strengths) had varying patterns of growth across farm samples. Microdilution plates showed growth in the wells apart from the double strength broth for one of the farm's sample.

The AST procedure for the EE broth samples was repeated as previously described, but transferring a 50µl aliquot of broth into 5ml of demineralised water instead of the exact amount needed for the standardised optical density. When checked

following the incubation period previously described, purity plates of the EE broths continued to show no visible bacterial growth. Bacterial growth in the wells of the microdilution plates were again difficult to interpret. Further repeats were carried out, but only on the EE broth sample from Farm A. A 'full strength dilution' was taken, whereby a 50µl aliquot of EE broth which was not diluted in any demineralised water was transferred into Mueller-Hinton broth. Additionally, a 50µl aliquot was added to 11.5ml of unused EE broth as a comparison for bacterial growth. For both the Mueller-Hinton and EE broth bacterial suspensions, all 96 wells of a microdilution plate were inoculated with 100µl of broth. Microdilution plate handling, including purity plating, was carried out as previously described. Following the incubation period, purity plates were checked with one colony of bacterial growth being identified when grown from Mueller-Hinton broth, but no visible growth was found for that using EE broth as a growth medium. Thereafter, the antimicrobial susceptibility testing procedure was repeated again for each EE broth sample, whereby 50µl of broth was transferred directly into Mueller-Hinton broth with no dilution in demineralised water. After incubation, it was found that there was growth on only one of the purity plates, but meaningful growth was now being seen in the microdilution plates.

A week after the first milk samples were received by the laboratory, bulk tank milk samples for 3 out of the 5 farms sampled initially were received for repeat testing. Processing of the raw milk samples was as described above, checked at 24 and 48 hours and plated as has been described. The AST procedure for the second round of broth samples was carried out using a 'full strength dilution' as well as dilutions meeting the standardised measure of optical density and then transferred into Mueller-Hinton broth. Purity plates were inoculated first with a 1µl loop as well as with a cotton swab to transfer more bacterial suspension from the positive control wells. Following incubation, visible growth on purity plates from EE broth samples were found when cotton swabs were used instead of 1µl loops, but only for the 'full strength' samples. Growth on purity plates was found for both AZ broths at 'full strength' when inoculated using both cotton swabs as well as 1µl loops. Growth on purity plates for broth samples at a standardised measure of optical density was

mixed, with no growth present for EE broth samples, with some growth for AZ broth samples. Growth in microdilution plate wells was best for 'full strength' samples.

5.2.1.4 Comparison of broth testing with agar testing

In addition to the AST procedure being carried out on the broths, this was also carried out from selective agar plates (TBX and SB) which had been plated from enrichment broth following their 48 hour incubation period. Standard AST procedures indicate the use of Columbia (5% sheep blood) agar in the culture of isolated bacterial specimens. In addition to testing the feasibility of an AST procedure directly from an enrichment broth procedure, eliminating the step of having to subculture isolates onto Columbia (5% sheep blood) agar would streamline the process. This was initially investigated by comparing the MICs of the bacterial species considered through the course of this thesis (*Enterococcus* spp. and *E. coli*) cultured on Columbia (5% of sheep blood) agar and their respective selective nutrient agars (SB and TBX).

Isolates to be investigated were selected from the dataset of bacterial MIC profiles generated in Chapter 2 and were recovered from frozen storage at -80°C (Protect Microorganism Preservation System, Technical Service Consultants Ltd, Heywood, UK). Isolates were plated onto their respective selective agars from glycerol beads upon which they were suspended. TBX plates were incubated at 44°C for 48 hours, while SB plates were initially incubated for 4 hours at 35°C, thereafter moved to incubate at 44°C for 44 hours. Following incubation, AST was carried out using Micronaut-S Mastitis 3 microdilution plates according to the procedure when using these plates which has been described previously for *Enterococcus* spp and *E. coli*. Results were then compared with MIC profiles obtained for previous AST as outlined in Chapter 2, where isolates were cultured on Columbia (5% sheep blood) agar.

Following these initial investigations, AST was carried out on the bacteria grown on the selective agars (SB and TBX) which had been plated from the enrichment broths following 48 hours of incubation. Colonies were identified morphologically, where growth of *Enterococcus* spp. appeared as red/maroon on SB plates and growth of *E. coli* as blue growth on TBX plates. Streak plates of 10µl were used, as separate

colonies on the 100µl spread plates were difficult to identify. A cotton swab was used to pick up portions of bacterial growth across the plates to ensure a representative spectrum of bacteria could be tested. This employed the assumptions made and tested in Chapter 4 that in a representative sample of more than one individual colony, the bacterial isolate which is most resistant to each of the antimicrobials tested will constitute the overall MIC profile. Following collection of bacteria from plates, the cotton swab was placed in the bottom of a vial containing 5ml of demineralised water and rotated to allow transfer of bacteria into the water. The optical density of the bacterial suspension was standardised using the Sensititre nephelometer. For *E. coli*, an aliquot of 50µl of bacterial suspension was added to 11.5ml of Mueller-Hinton broth and 100µl was added for *Enterococcus spp.* The inoculation, plate handling, incubation and reading procedures were carried out as previously described.

5.2.2 Application of established broth processing methodology

5.2.2.1 Farm recruitment and sampling

Bulk tank milk samples were sourced from a convenience sample of six farms local to the laboratory with pre-existing commercial and personal contacts. Bulk tank sampling occurred weekly across an 8 week period in the first quarter of 2022. Eight sampling kits consisting of four, 50ml universal tubes which were labelled with a farm number ID (1-6) and sampling week ID. Kits were delivered to farms by a member of staff from the laboratory. Bulk tank samples were taken prior to a morning milk collection (samples represented four milking events) following cooling and agitation of the bulk tank from the tank outlet. Following each instance of weekly sampling, samples were either returned to the laboratory by the farmer or collected by a member of staff. Once received by the laboratory, sample kits were labelled with the sampling date, and placed in a -20°C freezer until ready for processing.

5.2.2.2 Sample processing and antimicrobial susceptibility testing

A final overview of the sample processing procedure is provided in Figure 5.2 in section 5.2.3. following description here. Nutrient broths (EE and both strength AZ broths) were prepared as previously outlined (5.2.1.2). Broths were prepared in advance of sample processing occurring and were placed in cold storage until required.

Samples were processed in the order they were received by the laboratory. When ready for processing, two of the four 50ml universal tubes for each farm for a given sampling week were taken out of the freezer and allowed to defrost at room temperature. Enrichment broths required for the number of samples being processed were taken out of cold storage to bring them to room temperature. Once defrosted, the contents of each of the two 50ml universal tubes were transferred to a 100ml sterile container and inverted to allow mixing of the milk sample. For each farm's bulk tank milk sample, 25ml of milk were added to the 225ml portions of EE and both single and double strength AZ broths and incubated at 44°C. Broths were to be checked at 24 and 48 hours of incubation. Aliquots of raw milk samples were plated on SB, TBX and Columbia (5% sheep blood) agar plates as 10µl streak plates and 100µl spread plates. SB plates were incubated at 35°C for 4 hours then moved to incubate at 44°C for 44 hours, with TBX plates being incubated at 44° for 48 hours. Columbia (5% sheep blood) agar plates were incubated for 72 hours at 37°C.

At 24 hours of incubation, the broths were checked and taken out of incubation to be plated onto agar to examine how the microbiology of the raw milk samples were being enriched by selective broths. Aliquots of 10µl from each broth were streaked onto Columbia (5% sheep blood), TBX (from EE broth only) and SB (both strengths of AZ broth only). TBX and SB plates were incubated at 44°C for 48 hours with the Columbia (5% sheep blood) plates being incubated at 37°C for 72 hours. Broths were returned to incubation for another 24 hours, after which the same plating process was repeated.

Following the 48 hour broth incubation period, AST was undertaken and carried out according to the finalised process which was determined from the pilot study (5.2.1.3). For EE broths, a 50µl aliquot was added directly to 11.5 ml vials of Mueller Hinton broth, while 100µl aliquots were used for the two strengths of AZ broth. Bacterial suspensions were dispensed as 100µl aliquots into each well of the Micronaut-S Mastitis 3 microdilution plates. Purity plates were inoculated from the positive control wells using both a 1µl loop and a cotton swab. Microdilution and purity plates were handled and results read as has been outlined previously. In addition to testing performed on nutrient broths, for each sample comparisons were made with testing carried out on bacteria grown on selective agars (SB and TBX), the process of which has been outlined (5.1.2.4).

Once incubation for all agar plates was complete, bacterial colonies were identified by MALDI-TOF MS using Biotyper 3.1 (Bruker Daltonics, Coventry, UK). Bacterial colonies to be identified were selected based on varying morphologies across samples from all farms. Purity plates from enrichment broth testing were also identified according to MALDI-TOF MS to identify bacteria most likely to constitute the MIC profile. All results were collated for later reference.

5.2.2.2 Comparison of results between 24 hours and 48 hours of incubation

For all bulk tank milk samples, investigations of antimicrobial susceptibilities had been carried out at 48 hours. For the final three weeks of samples, these processes were carried out for samples enriched in broth and selectively isolated on agar at 24 hours of their incubation. This was to provide a comparison to investigate whether length of incubation had any effect on the MIC profiles of bacteria cultured for both of these processes. Methodologies in both cases were identical.

5.2.3 Overview of laboratory methodology

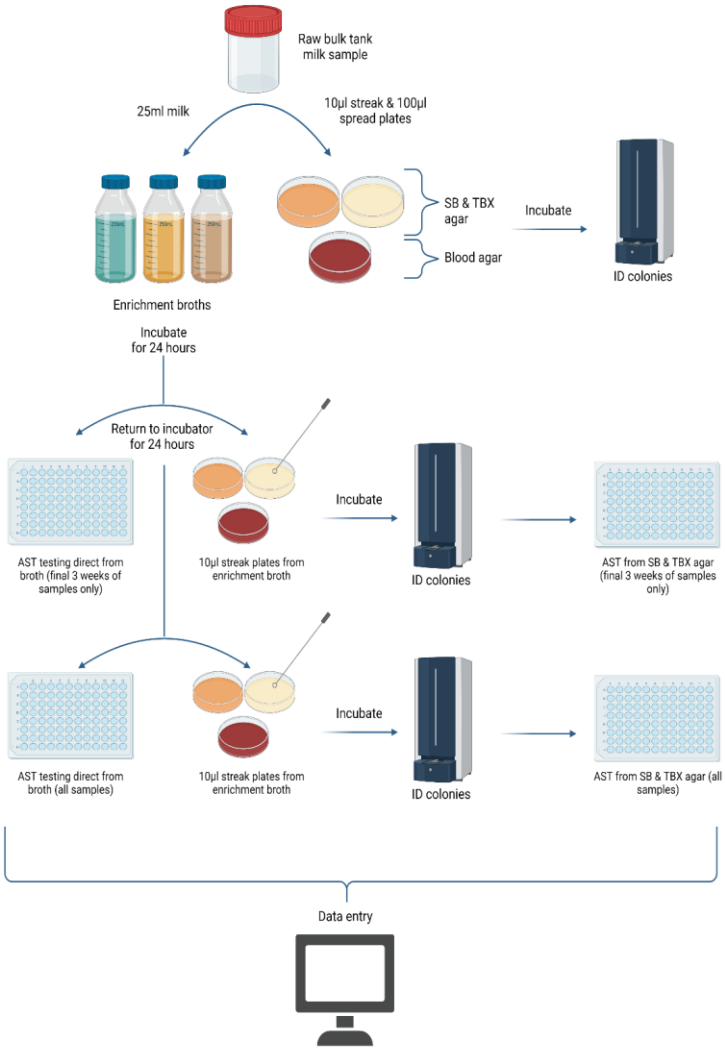


Figure 5.2. A diagrammatic overview of the steps involved for each bulk tank milk sample received for processing and determination of MICs. (Created with BioRender.com).

5.2.3 Data analysis

5.2.3.1 Descriptive analysis

Following completion of antimicrobial susceptibility testing, all data outputs were collated, sorted and cleaned. Data was inspected manually to identify: (i) potential trends in MIC profiles across the study period within and between farms and (ii) the degree of similarity of MIC profiles between the experimental broth enrichment method and the more standard agar culture method. Data were subsequently visualised graphically using the *ggplot2* package in R (Wickham, 2016). For this, MIC data points were rescaled from their tested concentration range to a standardised scale, allowing all MIC values to cover the same range. This approach was employed previously in Chapters 2 and 3 and was;

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

For each farm, three graphs were produced (one for each enrichment broth) which compared the MIC values between the two methods investigated for each antimicrobial tested and also displayed patterns of change across the 8 week study period.

5.2.3.2 Concordance analysis

Determination of concordance was measured across multiple areas which were investigated throughout the laboratory methodologies;

1. Bacterial MICs cultured on non-selective Columbia (5% sheep blood) agar and selective agars (SB for *Enteroccocus* spp. and TBX for *E. coli*).
2. MIC profiles derived from enrichment nutrient broths and corresponding selective agars at their respective 48 hours of incubation (and 24 hours for final 3 weeks of sampling)

3. Concordance of MICs generated from both strengths of AZ broth

Concordance between methods was measured via percentage agreement (PA);

$$PA = \frac{No.of\ measurements\ with\ same\ MIC\ value}{No.of\ total\ measurements\ observed}\ x\ 100$$

5.3 Results

5.3.1 Comparison of minimum inhibitory concentrations from agars from pilot study

During the pilot study, MICs of antimicrobials were compared when grown on selective nutrient agars (SB and TBX for *Enterococcus* spp. and *E. coli* respectively) with those grown on Columbia (5% sheep blood) agar. Table 5.1 provides an overview of percentage agreement for these comparisons. Concordance between MICs for antimicrobials when cultured on SB and TBX compared with Columbia (5% sheep blood) agar was 100% for 7 and 6 out of 11 antimicrobials tested respectively. However, when checked manually for ± 1 microdilutions following calculation of percentage agreement of raw values, all antimicrobials across both comparisons had 100% agreement. Only one isolate of *E. coli* tested did not conform to this, where the difference in MIC for ampicillin, cefazolin, cefoperazon and cefquinome was greater than one microdilution.

Table 5.1~~5.1~~. An outline of concordance measured by percentage agreement for comparisons of MICs made between *Enterococcus* spp. and *E. coli* isolates cultured on SB and TBX agar with the same isolates which had been cultured on Columbia (5% sheep blood). Twelve isolates of each bacterial species were used in the two comparisons.

Antimicrobial	SB (comparisons; n=12).	TBX (comparisons; n=12).
Amoxicillin Clavulanic acid	100	100
Ampicillin	100	94.4
Cefazolin	50	94.4
Cefoperazon	100	94.4
Cefquinome	66	88.9
Erythromycin	100	100
Kanamycin/Cefalexin	100	88.9
Marbofloxacin	66	100
Oxacillin	100	100
Penicillin G	33.3	100
Pirlimycin	100	100

5.3.2 Identification of bacteria cultured from raw milk as part of pilot study

Results of identification of bacteria cultured across a range of agar types provided information on the selectivity of enrichment broths. Table 5.2 provides an overview of bacteria cultured on Columbia (5% sheep blood), Edward's, MacConkey, SB and TBX agars from raw milk from bulk tank samples analysed from pilot study farms. Table 5.3 and Table 5.4 provide an overview of bacteria identified following the plating procedures from enrichment broth after 24 and 48 hours of incubation respectively.

Culture of raw milk highlighted a diversity of bacterial species present. Staphylococci were frequently identified, as well as *Pseudomonas aeruginosa*, *Lactococcus* spp., *Streptococcus* spp. as well as *E. coli* and *Enterococcus* spp. The diversity of bacterial species identified may highlight a host of others which were not chosen for identification via MALDI-TOF MS.

Nutrient broths were found to enrich raw bulk tank milk samples. At 24 hours of incubation, individual *E. coli* colonies were more easily identified when plated on non-selective agar from EE broth. On TBX agar, a greater density of *E. coli* colonies were found, showing the nutrient broth was amplifying the population of *E. coli* in milk samples. However, other bacterial species were also cultured across non-selective

and selective agars. The most common of these were *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. For both strengths of AZ broth, it was found that the population of *Enterococcus* spp. from raw milk had been enriched when aliquots of broth had been plated. However, on non-selective agar, other bacterial species were still found to be cultured.

Plating aliquots of enrichment broth at 48 hours of incubation yielded similar results in terms of bacterial species identified. Although individual colonies of *E. coli* and *Enterococcus* spp. could still be easily identified and isolated, growth of other bacterial species still featured on non-selective agar. This demonstrated that although both EE and AZ (single and double strength) broths were enriching *E. coli* and *Enterococcus* spp. populations, other bacterial species that were not of interest were also being enriched.

Table 5.2~~5.2~~. An overview of bacterial colonies selected and identified from plating raw bulk tank milk samples from pilot study farms (n=5) received by the laboratory prior to enrichment by nutrient broths.

Farm	Nutrient Agars				
	Columbia (5% sheep blood)	**Edward's agar**	**MacConkey**	**SB**	**TBX**
1	*Lactococcus lactis*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*
	Staphylococcus haemolyticus	*Streptococcus uberis*	*Moraxella osloensis*		*Escherichia coli*
	Kocuria salsicia	*Staphylococcus* spp.	*Staphylococcus xylosus*		
2	*Staphylococcus chromogenes*	*Aerococcus viridans*	*Staphylococcus sciuri*	*Enterococcus faecium*	*Escherichia coli*
	Staphylococcus auereus	*Enterococcus faecalis*	*Staphylococcus aureus*		*Lactobacillus* spp.
		Lactococcus garvieae	*Pseudomonas oleovorans*		
3	*Acinetobacter jonhsonii*	*Enterococcus casseliflavus*	*Pseudomonas aeruginosa*	*Enterococcus faecium*	*Pseudomonas aeruginosa*
	Pseudomonas aeruginosa	*Lactococcus lactis*	*Klebsiella oxytoca*		*Enterobacter cloacae*
		Streptococcus dysgalactiae	*Pseudomonas monteilii*		*Escherichia coli*
4	*Staphylococcus chromogenes*	*Aerococcus viridans*	*Staphylococcus chromogenes*	*Enterococcus faecalis*	*Escherichia coli*
	Staphylococcus aureus	*Staphylococcus simulans*	*Staphylococcus haemolyticus*	*Enterococcus faecium*	
			Staphylococcus equorum		
5	*Bacillus licheniformis*	*Enterococcus faecalis*	*Bacillus pumilus*	*Enterococcus faecalis*	*Escherichia coli*
	Escherichia coli	*Enterococcus durans*	*Escherichia coli*		
	Staphylococcus chromogenes				

Table 5.3~~5.3~~. An overview of bacteria identified from plating enrichment broths at 24 hours of incubation from pilot study farms (n=5). Bacteria were cultured on non-selective (Columbia (5% sheep blood) agar from EE and AZ (both strengths) broths and on selective agar (EE broth streaked on TBX and AZ broths on SB) agar.

Farm	24 hr Broth Non-Selective Agar			24 hr Broth Selective Agar		
	EE	**Single strength AZ**	**Double strength AZ**	**EE**	**Single strength AZ**	**Double strength AZ**
1	*Pseudomonas aeruginosa*	*Enterococcus faecium*	*Enterococcus faecium*	*E. coli*	*Enterococcus faecium*	*Enterococcus faecium*
	Staphylococcus warneri		*Enterococcus hirae*	*Pseudomonas aeruginosa*		*Enterococcus hirae*
	E. coli					
2	*Bacillus horikoshii*	*Candida krusei*	*Staphylococcus haemolyticus*	*E. coli*	*Enterococcus faecium*	*Enterococcus hirae*
	E. coli	*Enterococcus faecium*	*Candida krusei*	*Klebsiella pneumoniae*		
	Pseudomonas aeruginosa		*Enterococcus faecium*			
3	*Pseudomonas aeruginosa*	*Staphylococcus warneri*	*Kocuria rhizophila*	*E coli*	*Enterococcus faecium*	*Enterococcus faecium*
	E. coli	*Enterococcus faecium*	*Lactobacillus gasseri*	*Pseudomonas aeruginosa*		
			Enterococcus faecium			
4	*E. coli*	*Staphylococcus chromogenes*	*Enterococcus hirae*	*E coli*	*Enterococcus faecalis*	*Enterococcus hirae*
		Enterococcus faecalis		*Klebsiella pneumoniae*		
5	*E. coli*	*Enterococcus faecalis*	*Enterococcus faecalis*	*E coli*	*Enterococcus faecium*	*Enterococcus faecium*
		Streptococcus alatolyticus				

Table 5.4~~5.4~~. An overview of bacteria identified from plating enrichment broths at 48 hours of incubation from pilot study farms (n=5). Bacteria were cultured on non-selective (Columbia (5% sheep blood) agar from EE and AZ (both strengths) broths and on selective agar (EE broth streaked on TBX and AZ broths on SB) agar.

Farm	48 hr Broth Non-Selective Agar			48 hr Broth Selective Agar		
	EE	**Single strength AZ**	**Double strength AZ**	**EE**	**Single strength AZ**	**Double strength AZ**
1	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Neisseria macacae*	*E coli*	*Enterococcus faecium*	*Enterococcus faecium*
	E. coli	*Lactobacillus johnsonii*	*Staphylococcus epidermis*	*Klebsiella pneumoniae*		
			Enterococcus hirae			
2	*E. coli*	*Enterococcus faecium*	*Enterococcus faecalis*	*E .coli*	*Enterococcus faecium*	*Enterococcus faecium*
3	*E. coli*	*Enterococcus faecium*	*Lactobacillus gasseri*	*E. coli*	*Enterococcus faecium*	*Enterococcus faecium*
			Enterococcus faecium			
4	*Klebsiella pneumoniae*	*Staphylococcus haemolyticus*	*Enterococcus hirae*	*E coli*	*Enterococcus faecalis*	*Enterococcus hirae*
	E. coli	*Enterococcus faecalis*				
5	*E. coli*	*Enterococcus faecalis*	*Enterococcus faecalis*	*E coli*	*Enterococcus faecium*	*Enterococcus faecium*
		Lactobacillus fermentum	*Enterococcus hirae*		*Enterococcus faecalis*	

5.3.3 Descriptive overview of minimum inhibitory concentration profiles

A total of 48 bulk tank milk samples were received and analysed. Table 5.5 to Table 5.10 provide an overview of the MIC profiles obtained for Farms 1-6 across the 8 weeks of sampling for both EE (enrichment broth) and TBX (selective agar). Bacterial IDs obtained from purity plates taken as part of enrichment broth AST testing are included. For antimicrobials tested against EE broth, penicillin G, oxacillin, pirlimycin and erythromycin consistently had MICs which corresponded to the highest microdilutions measured on the test plate across all 8 weeks for all farms. Specific differences were observed between farms. Farm 1 had low MICs for all other antimicrobials across most weeks tested, with heightened values for some weeks mainly observed for ampicillin. Farm 2 had the lowest MICs for all other antimicrobials tested, while Farm 3 had increases in MIC values for ampicillin and kanamycin/cefalexin for weeks 5 and 6 before returning to lower values for the final sampling weeks. Fluctuations in the MIC for amoxicillin/clavulanic acid, ampicillin, cefazolin, kanamycin/cefalexin and marbofloxacin were observed for Farm 4. Relatively low MICs were observed for Farms 5 and 6, but with consistently high MICs for ampicillin for Farm 6, whereas this was observed in weeks 1-3 and week 8 for Farm 5.

MICs obtained when testing *E. coli* which were selected for from TBX agar had less variation across the sampling weeks when compared to that for EE broth, but consistently high MICs for penicillin G, oxacillin, pirlimycin and erythromycin were also observed. MIC values for farms 1 and 2 were consistently low across the sampling period, although with higher MICs for ampicillin in weeks 1 and 8 and for marbofloxacin in week 1 for the former. Across all farms, most disruption to the pattern of mostly low MICs were observed for amoxicillin/clavulanic acid, ampicillin and kanamycin/cefalexin.

Table 5.11 to Table 5.16 provide an overview of this data obtained for AZ broth (single strength) and SB agar on which the broth was plated.). Bacterial IDs obtained from purity plates taken as part of enrichment broth AST testing are included. MIC values from broth testing showed decreased variation both between farms and across the

sampling period for each farm. Amoxicillin/clavulanic acid and ampicillin were consistently measured low, with the converse observed for the cephalosporin antimicrobials tested, however some variation was observed for cefquinome. Most of the variation displayed between farms and across sampling weeks was observed for erythromycin and marbofloxacin, with more acute variation found for kanamycin/cefalexin, penicillin G and pirlimycin. When compared with results for *Enterococcus* spp. selectively tested from SB plates, similar patterns were observed.

Table 5.17 to Table 5.22 provide this data for double strength AZ broth and SB agar on which the broth was plated. Bacterial IDs obtained from purity plates taken as part of enrichment broth AST testing are included. Due to time constraints and supply issues with microdilution plates, antimicrobial susceptibility testing from *Enterococcus* spp. selected for from SB agar plates streaked from double strength AZ broth was only carried out for the final three weeks of sampling. MIC profiles generated from double strength AZ broth were somewhat similar to results obtained for single strength AZ broth. However, some marked differences were observed. Heightened MICs were found for amoxicillin/clavulanic acid and ampicillin for Farms 1, 3 and 5 for select weeks between sampling weeks 1-5. Where changes in MIC values derived from broth occurred, these were largely mirrored by those derived from agar plates.

Table 5.5~~5.5~~. Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 1** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 1 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	*E. coli, K. aerogenes*	>32	<=4	>16	>16	>32	<=4	16	<=2	<=1	<=1	>4	>4	16	<=4	>2	>2	>4	>4	>8	>8	>4	>4
Week 2	*E. coli*	<=4	<=4	>16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	>2	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	K. *pneumoniae*	32	<=4	16	<=4	16	<=4	8	<=2	<=1	<=1	>4	>4	16	<=4	0.5	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 7	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 8	*E.coli*	<=4	<=4	16	8	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4

Table 5.6~~5.6~~. Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 2** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 2 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	*E. coli*	8	<=4	<=4	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 2	*E. coli*	<=4	<=4	8	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 7	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	8	>8	>4	>4
Week 8	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4

Table 5.7~~5.7~~. Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 3** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 3 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	H. *alvei*, *E. coli*	8	<=4	16	>16	32	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 2	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*E. coli*	<=4	<=4	16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	*E. coli*	<=4	<=4	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	>32	>32	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	8	<=4	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	32	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 7	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>8	>4	>8	>8	>4	>4
Week 8	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4

Table 5.8~~5.8.~~ Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 4** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 4 Sampling week	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	*P. aeruginosa*	32	>32	>16	>16	32	>32	<=2	<=2	<=1	<=1	>4	>4	8	16	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 2	*E. coli*	<=4	<=4	<=4	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*P. aeruginosa*	>32	>32	>16	>16	>32	>32	<=2	<=2	<=1	<=1	>4	>4	16	32	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*P. aeruginosa*	<=4	<=4	>16	<=4	>32	<=4	4	<=2	2	<=1	>4	>4	16	<=4	0.5	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	*E. coli*	<=4	<=4	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	<=4	<=4	8	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 7	*E. coli*	8	8	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	16	16	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 8	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	4	>4	8	<=4	0.5	<=0.25	4	>4	>8	>8	>4	>4

Table 5.9~~5.9~~. Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 5** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 5 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	*S. saprophyticus* *E. coli*	8	8	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 2	*E. coli*	8	<=4	>16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*E. coli*	<=4	<=4	>16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	8	>8	>4	>4
Week 7	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 8	*E. coli*	<=4	<=4	16	<=4	<=4	<=4	<=4	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4

Table 5.10~~5.10~~. Raw MIC values measured direct from EE broth and TBX agar (streaked from EE broth) following 48 hours of incubation for **Farm 6** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 6 Sampling week	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		EE	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**	**EE**	**TBX**
Week 1	*E. coli*	8	8	>16	>16	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 2	*P. aeruginosa*	8	<=4	>16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 3	*E. coli*	<=4	<=4	>16	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 4	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 5	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 6	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	8	>8	>4	>4
Week 7	*E. coli*	<=4	<=4	<=4	<=4	<=4	<=4	<=2	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4
Week 8	*E. coli*	<=4	<=4	16	<=4	<=4	<=4	<=4	<=2	<=1	<=1	>4	>4	<=4	<=4	<=0.25	<=0.25	>4	>4	>8	>8	>4	>4

Table 5.11~~5.11~~. Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 1** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 1 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	E. *faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	2	4	>32	>32	>2	>2	>4	>4	>8	4	>4	>4
Week 2	E. *faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	0.5	1	>32	>32	>2	>2	>4	>4	2	4	2	>4
Week 3	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	>4	>32	>32	>2	>2	>4	>4	8	8	>4	<=1
Week 4	E. *faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	16	>8	8	0.5	1	>32	8	2	1	>4	>4	2	2	4	>4
Week 5	E. *faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	0.5	0.5	>32	>32	>2	2	>4	>4	4	2	4	>4
Week 6	E. *faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	>4	>32	>32	>2	>2	>4	>4	4	2	>4	>4
Week 7	E. *faecium*	<=4	<=4	<=4	<=4	32	>32	16	16	4	2	0.5	2	32	32	>2	>2	4	4	1	1	4	>4
Week 8	E. *faecium*	<=4	<=4	<=4	<=4	32	32	16	8	<=1	<=1	0.5	<=0.125	>32	>32	2	2	4	>4	0.5	2	>4	>4

Table 5.12~~5.12~~. Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 2** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 2 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	E. *hirae*	<=4	<=4	<=4	<=4	>32	>32	16	16	<=1	<=1	2	4	32	32	2	2	>4	>4	2	4	>4	>4
Week 2	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	8	4	>4	>32	>32	>2	>2	>4	>4	2	4	>4	<=1
Week 3	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	16	>16	<=1	8	2	0.5	32	>32	1	2	>4	>4	2	2	>4	>4
Week 4	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	<=0.125	<=0.125	>32	>32	2	2	>4	>4	2	1	2	<=1
Week 5	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	8	8	>4	4	>32	>32	>2	2	>4	>4	4	4	>4	>4
Week 6	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	4	>4	>32	>32	>2	2	>4	>4	4	8	<=1	<=1
Week 7	L. *delbrueckii* E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	2	4	4	4	>32	>32	1	>2	>4	>4	4	4	2	>4
Week 8	E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	16	8	4	<=1	4	1	>32	32	2	1	>4	>4	1	1	4	<=1

Table 5.13~~5.13~~. Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 3** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 3 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	S. *capitis* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	0.25	>32	>32	2	>2	>4	>4	4	2	>4	2
Week 2	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	2	1	>4	>4	4	4	>4	>4
Week 3	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	16	16	>8	>8	2	1	>32	>32	<=0.25	>2	>4	>4	4	1	>4	2
Week 4	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	16	8	<=1	4	<=0.125	>32	16	2	1	>4	>4	4	2	>4	<=1
Week 5	*P. acidlactii* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	2	>4	4	>32	32	1	1	>4	>4	>8	2	>4	<=1
Week 6	*L. fermentum* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	2	2	>4	>4	4	4	>4	<=1
Week 7	L. *delbrueckii* E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	16	>8	4	>4	4	>32	32	>2	1	>4	>4	4	2	>4	<=1
Week 8	*E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	16	16	<=1	<=1	0.5	<=0.125	16	16	1	2	>4	>4	2	4	<=1	<=1

Table 5.14~~5.14~~. Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 4** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 4 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecalis* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	16	2	>8	1	4	>32	32	>2	>2	>4	>4	2	1	<=1	<=1
Week 2	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	16	16	8	>8	2	>4	16	32	>2	>2	>4	>4	2	2	<=1	<=1
Week 3	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	2	>4	32	32	>2	>2	>4	>4	2	2	>4	>4
Week 4	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	2	>32	>32	>2	>2	>4	>4	4	2	>4	>4
Week 5	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	2	2	>32	>32	>2	>2	>4	>4	2	4	2	4
Week 6	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	0.25	>32	>32	>2	>2	>4	>4	2	2	>4	>4
Week 7	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	16	>16	4	>8	1	0.25	32	>32	>2	>2	4	>4	1	2	>4	>4
Week 8	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	0.25	0.25	>32	>32	>2	2	>4	>4	2	4	2	4

Table 5.15~~5.15~~**.** Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 5** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 5 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	4	>8	0.5	4	32	>32	2	>2	>4	>4	4	4	<=1	<=1
Week 2	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	16	>16	<=1	2	0.5	4	>32	>32	2	2	>4	>4	1	2	>4	>4
Week 3	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	4	>32	>32	>2	>2	>4	>4	8	8	>4	>4
Week 4	*M. luteus* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	>2	>2	>4	>4	8	8	>4	>4
Week 5	*E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	>4	>32	>32	2	>2	>4	>4	8	8	>4	>4
Week 6	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	>2	>2	>4	>4	8	>8	>4	>4
Week 7	L. *delbrueckii* E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	4	>4	>32	>32	>2	>2	>4	>4	8	>8	>4	>4
Week 8	L. *delbrueckii* E. *faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	1	2	>4	>4	4	8	>4	>4

Table 5.16~~5.16~~. Raw MIC values measured direct from single strength AZ broth and SB agar (streaked from single strength AZ broth) following 48 hours of incubation for **Farm 6** for each week throughout the 8 week sampling period (n= 48). Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 6 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. hirae*	<=4	<=4	<=4	<=4	>32	>32	8	16	>8	>8	>4	<=1	16	16	2	1	>4	>4	2	2	>4	>4
Week 2	*E. hirae*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	4	32	>32	>2	>2	>4	>4	2	2	>4	>4
Week 3	*E. hirae* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	2	>32	32	>2	>2	>4	>4	8	2	>4	>4
Week 4	*E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	2	32	16	>2	>2	>4	>4	2	2	>4	>4
Week 5	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	1	32	32	>2	>2	>4	>4	2	2	>4	>4
Week 6	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	16	8	>8	2	4	>32	>32	>2	>2	>4	>4	2	2	>4	>4
Week 7	*E. hirae* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	16	16	4	>8	1	1	33	32	>2	>2	4	>4	1	8	>4	>4
Week 8	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	16	16	>8	>8	1	1	>32	16	>2	>2	>4	>4	1	2	2	>4

Table 5.17~~5.17~~**.** Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 1** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 1 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecalis*	<=4		8		>32		>16		>8		2		>32		>2		>4		>8		2	
Week 2	*E. faecium* *E. faecalis*	<=4		<=4		>32		>16		>8		2		>32		>2		>4		4		4	
Week 3	*E. faecium*	<=4		>16		>32		>16		>8		1		>32		2		>4		>8		>4	
Week 4	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		4		>4	
Week 5	*E. faecalis* *E. durans*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 6	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	8	16	<=1	4	1	>4	>32	>32	>2	>2	4	>4	0.5	4	>4	>4
Week 7	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	8	16	2	>8	2	>4	>32	>32	>2	>2	4	>4	1	2	>4	>4
Week 8	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	4	>32	>32	2	>2	>4	>4	4	>8	<=1	>4

Table 5.18~~5.18~~**.** Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 2** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 2 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 2	*E. hirae*	<=4		<=4		>32		16		<=1		<=0.125		>32		1		>4		2		4	
Week 3	*E. faecium*	<=4		<=4		>32		>16		>8		1		>32		>2		>4		2		>4	
Week 4	*E. faecium*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		4		>4	
Week 5	*S. hominis* *E. hirae*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 6	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	>2	>2	>4	>4	4	4	>4	<=1
Week 7	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	8	>4	4	>32	>32	>2	2	>4	>4	4	4	2	<=1
Week 8	*E. durans*	<=4	<=4	<=4	<=4	32	>32	8	16	<=1	<=1	1	<=0.125	8	8	1	1	>4	4	0.25	1	<=1	<=1

Table 5.19~~5.19~~. Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 3** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 3 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		2		>4		4		>4	
Week 2	*E. faecium*	>32		<=4		>32		>16		>8		>4		>32		>2		>4		4		>4	
Week 3	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		8		>4	
Week 4	*E. faecium*	<=4		<=4		>32		>16		>8		>4		>32		2		>4		8		>4	
Week 5	*E. hirae* *M. osloensis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		4		>4	
Week 6	*E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	16	>8	4	>4	>4	>32	>32	>2	>2	>4	>4	4	2	>4	>4
Week 7	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	16	16	>8	<=1	>4	4	>32	>32	2	1	>4	>4	4	1	>4	<=1
Week 8	*E. faecium* *E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	2	2	>4	>4	4	4	>4	>4

Table 5.20~~5.20~~. Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 4** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 4 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*L. fermentum* *E. faecalis*	<=4		<=4		>32		<=2		<=1		4		<=4		2		>4		0.5		>4	
Week 2	*E. faecium* *S. gallolyticus*	<=4		<=4		>32		>16		>8		4		>32		>2		>4		2		>4	
Week 3	*E. faecium*	<=4		<=4		>32		>16		>8		1		>32		>2		>4		4		>4	
Week 4	*E. faecium*	<=4		<=4		>32		>16		>8		2		>32		>2		>4		4		4	
Week 5	*E. faecalis* *E. faecium*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 6	*E. faecium* *L. fermentum*	<=4	<=4	<=4	<=4	32	>32	16	8	4	8	2	2	32	8	>2	>2	>4	>4	1	1	2	>4
Week 7	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	4	<=0.125	>32	>32	>2	2	>4	>4	2	2	>4	>4
Week 8	*E. faecalis*	<=4	<=4	<=4	<=4	16	>32	16	>16	<=1	>8	0.5	>4	32	32	>2	2	4	>4	1	4	<=1	>4

Table 5.21~~5.21~~. Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 5** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 5 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. faecalis*	>32		<=4		>32		>16		8		>4		>32		>2		>4		8		>4	
Week 2	*E. faecium*	>32		<=4		>32		>16		>8		>4		>32		>2		>4		8		>4	
Week 3	*E. faecalis*	>32		>16		>32		>16		>8		>4		>32		>2		>4		8		>4	
Week 4	*E. faecalis*	>32		<=4		>32		>16		>8		>4		>32		>2		>4		8		>4	
Week 5	*E. faecium*	>32		>16		>32		>16		>8		>4		>32		>2		>4		>8		>4	
Week 6	*E. faecalis* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	>2	>2	>4	>4	8	>8	>4	>4
Week 7	*E. faecalis*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	4	4	>32	>32	>2	2	>4	>4	8	2	>4	>4
Week 8	*E. faecalis* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	>4	>4	>32	>32	>2	2	>4	>4	8	8	>4	>4

Table 5.22~~5.22~~**.** Raw MIC values measured direct from double strength AZ broth and SB agar (streaked from double strength AZ broth) following 48 hours of incubation for **Farm 6** for each week throughout the 8 week sampling period (n= 48). MICs were not determined from SB agar from between Weeks 1 and 5. Bacteria identified from broth testing pure plates are presented.

		Antimicrobials																					
Farm 6 **Sampling week**	**Broth Test Purity Plate ID**	**Amoxicillin Clavulanic acid**		**Ampicillin**		**Cefazolin**		**Cefoperazon**		**Cefquinome**		**Erythromycin**		**Kanamycin/ Cefalexin**		**Marbofloxacin**		**Oxacillin**		**Penicillin G**		**Pirlimycin**	
		SS	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**	**SS**	**SB**
Week 1	*E. hirae*	<=4		<=4		>32		>16		>8		1		>32		>2		>4		1		>4	
Week 2	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		32		>2		>4		2		>4	
Week 3	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 4	*E. faecalis* *E. faecium*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		2		>4	
Week 5	*E. faecalis*	<=4		<=4		>32		>16		>8		>4		>32		>2		>4		4		>4	
Week 6	*E. faecalis* *E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	16	>8	>8	1	>4	>32	>32	>2	>2	>4	>4	2	2	>4	>4
Week 7	*E. faecium* *E. hirae*	<=4	<=4	<=4	<=4	>32	>32	>16	>16	>8	>8	1	2	>32	32	>2	>2	>4	>4	2	2	>4	>4
Week 8	*E. faecium*	<=4	<=4	<=4	<=4	>32	>32	>16	16	>8	>8	1	1	>32	32	>2	>2	>4	>4	4	2	>4	>4

5.3.4 Comparison of enrichment broth methodology with agar testing

Measures of concordance via calculation of percentage agreement between MICs generated from direct enrichment broth testing and those obtained by testing bacterial colonies cultured on selective agar for EE broth and TBX agar, single strength AZ broth and SB agar and double strength AZ broth and SB agar are provided in Table 5.23.

Table 5.23~~5.23~~. An outline of concordance measured by percentage agreement of MICs for each antimicrobial tested for between investigative methods (enrichment broth and agar culture) across 8 weeks of sampling (n= 48).

Antimicrobial	**EE/TBX**	**Single Strength AZ/ SB**	**Double strength AZ/ SB**
Amoxicillin/Clavulanic acid	80.9	100	100
Ampicillin	70.2	100	100
Cefazolin	87.2	97.8	83.3
Cefoperazon	93.6	77.8	33.3
Cefquinome	95.7	66.7	44.4
Erythromycin	97.9	28.9	27.8
Kanamycin/Cefalexin	83	68.9	66.7
Marbofloxacin	91.5	60	50
Oxacillin	93.8	95.6	77.8
Penicillin G	95.7	46.7	11.1
Pirlimycin	100	71.1	50

Percentage agreement between MICs determined from direct AST of enrichment broth with that of *E. coli* colonies selected for AST from TBX agar was generally high, with the lowest being 70.2% agreement for ampicillin. For the remaining 10 antimicrobials, percentage agreement was >80%. There was complete agreement between methods for pirlimycin. Concordance between AZ broths and their respective SB plates was much less than that for EE/TBX. Amoxicillin/clavulanic acid and ampicillin both had 100% agreement, with cefazolin being the next highest antimicrobial for agreement for both AZ broth comparisons. Lowest levels of agreement for both was found for erythromycin and penicillin G, with the latter showing only 11.1% agreement for concordance between double strength AZ broth and respective SB agar comparison.

However, when raw MIC values were checked manually for ± one microdilution difference (when initial concordance was measured to be <90%) between the broth testing and agar testing method measurements, levels of agreement were much improved. Table 5.24 provides an overview of concordance as measured by percentage agreement following this re-evaluation of raw MICs.

Table 5.24~~5.24~~. An outline of concordance measured by percentage agreement of MICs for each antimicrobial tested for between investigative methods (enrichment broth and agar culture) across 8 weeks of sampling (n= 48) following manual checking of MICs for ± microdilution measures.

Antimicrobial	**EE/TBX**	**Single Strength AZ/ SB**	**Double strength AZ/ SB**
Amoxicillin/Clavulanic acid	93.6	100	100
Ampicillin	78.7	100	100
Cefazolin	89.4	97.8	94.4
Cefoperazon	93.6	100	88.8
Cefquinome	95.7	82.2	66.6
Erythromycin	97.9	82	61
Kanamycin/Cefalexin	89.4	97.7	83.3
Marbofloxacin	91.5	95	88.8
Oxacillin	93.8	95.6	100
Penicillin G	95.7	93.3	83.3
Pirlimycin	100	100	61

Following re-evaluation to allow for a difference in microdilution of one point on the scale, percentage agreement for antimicrobials improved for all three comparisons being made. For comparisons between EE and TBX, eight out of eleven antimicrobials had ≥ 90% agreement. For single strength AZ and SB, this was nine out of eleven antimicrobials and for double strength AZ and SB, this was seen for only four antimicrobials.

5.3.5 Comparisons of minimum inhibitory concentrations at 24 and 48 hours

As enrichment broths had been incubated for 24 hours, checked, plated onto agar and incubated for a further 24 hours, antimicrobial susceptibility testing was carried out at 24 hours (in addition to testing at 48 hours) for the final three weeks samples were received. Comparisons were made between both antimicrobial susceptibility testing time periods for each of the three enrichment broths and each of the three

types of agar culture (plated from respective enrichment broths). An overview of concordance between 24 and 48 hours as measured by percentage agreement are presented in Table 5.25 and Table 5.26 respectively. Results are presented as raw MIC values before being checked for ± 1 microdilution, with percentage agreement following checking of the data being presented alongside.

With regards to concordance between MICs between incubation times for enrichment broths, agreement was mostly high for EE broth with all but three antimicrobials having > 80% agreement. Ampicillin had the worst concordance with 58.8%. Following re-evaluation to consider ± microdilution, concordance increased for all antimicrobials apart from cefazolin and marbofloxacin. For both of the AZ broth strengths, concordance varied greatly amongst antimicrobials. For both, penicillin G had the poorest agreement between AST events, with only 16.7% agreement for both single and double strength. Following manual checking of raw MIC values, concordance increased for all antimicrobials, apart from erythromycin for double strength AZ.

Table 5.25~~5.25~~. An outline of concordance measured by percentage agreement of MICs generated from AST for each antimicrobial tested for enrichment broths investigated at 24 and 48 hours of incubation for the final 3 weeks of sampling (n= 18). Concordance is presented for raw MIC outputs as well as manual checks for ± 1 microdilution measures.

Antimicrobial	EE		Single Strength AZ		Double strength AZ	
	Raw MIC	Checked MIC	Raw MIC	Checked MIC	Raw MIC	Checked MIC
Amoxicillin Clavulanic acid	76.5	82.4	100	-	100	-
Ampicillin	58.8	76.5	100	-	94.4	100
Cefazolin	76.5	76.5	88.9	100	88.9	100
Cefoperazon	100	-	61.1	100	58.3	91.6
Cefquinome	100	-	55.6	61.1	66.7	75
Erythromycin	94.1	100	33.3	72.2	50	50
Kanamycin/Cefalexin	82.4	100	83.3	94.4	75	91.6
Marbofloxacin	88.2	88.2	61.1	88.8	75	91.6
Oxacillin	94.1	100	77.8	100	91.7	100
Penicillin G	82.4	100	16.7	77.7	16.7	66
Pirlimycin	100	-	66.7	100	66.7	100

Concordance between AST events from selective agars was higher than that for enrichment broths. For TBX, eight out of eleven antimicrobials had percentage agreement ≥ 90%, which remained unchanged following manual re-evaluation

despite improved concordances being observed. For SB agar plated from single strength AZ broth, following checking ten out of eleven antimicrobials had ≥ 90% agreement, while six were observed as having this level of concordance for SB agar plated from double strength AZ broth.

Table 5.26~~5.26~~. An outline of concordance measured by percentage agreement of MICs generated from AST for each antimicrobial tested following agar culturing from enrichment broth plating, investigated at 24 and 48 hours of incubation for the final 3 weeks of sampling (n= 18). Concordance is presented for raw MIC outputs as well as manual checks for ± 1 microdilution measures

Antimicrobial	TBX		Single Strength AZ SB		Double strength AZ SB	
	Raw MIC	Checked MIC	Raw MIC	Checked MIC	Raw MIC	Checked MIC
Amoxicillin Clavulanic acid	80	86.6	100	-	100	-
Ampicillin	73.3	80	100	-	100	-
Cefazolin	93.3	-	92.9	-	91.7	-
Cefoperazon	100	-	78.6	100	58.3	85.7
Cefquinome	100	-	57.1	85.7	50	66.6
Erythromycin	100	-	64.3	100	58.3	69.2
Kanamycin/Cefalexin	86.7	86.7	71.4	100	66.7	84.6
Marbofloxacin	93.3	-	64.3	100	66.7	100
Oxacillin	100	-	92.9	-	75	91.6
Penicillin G	100	-	35.7	92.9	41.7	83.3
Pirlimycin	100	-	100	-	83.3	100

5.4 Discussion

AST according to BMD is a multi-step process involving culture of bacteria of interest from samples, preparation and standardisation of the inoculum, inoculation and incubation of test plates and reading of results (Bayot and Bragg, 2022). In the case of the AST procedure carried out and described in Chapter 2, there existed a significant processing time from the point of bulk tank milk sample receipt, culture of individual bacterial isolates, subculture onto agar and subsequent AST. The primary aim of the investigations outlined in this Chapter sought to investigate the viability of AST of bulk tank milk samples direct from enrichment broth culture. Such an investigation also allowed for consideration of regular sampling of bulk tank milk to screen for changes in bacterial MICs over time to assess the value of potential monitoring tool. The basis for these investigations lay in the conclusions made in

Chapter 4, whereby a single MIC as a measure of a farm's level of antimicrobial resistance was found to be largely representative of the highest MIC across a mixed bacterial population.

Initial investigations considered the effect of culturing bacterial isolates on selective agars, in this case SB for *Enterococcus* spp. and TBX for *E. coli*, prior to AST. Standardised methods determining antimicrobial susceptibilities utilise non selective agars, such as Mueller Hinton agar in Kirby Bauer disc diffusion (Hudzicki, 2009) and Columbia (5% sheep blood) agar as specified by the Thermofisher Sensititre procedure. The ability to carry out AST from bacteria cultured from environmental samples on selective agar would reduce the need for sub culturing onto a non-selective agar such as Columbia (5% sheep blood) agar as part of a standard protocol. Bayot and Bragg (2022) claimed that nonconformity with standard AST processes could impact the overall reliability of testing outcomes. This highlighted the need to investigate any deviation from any standards. Comparisons of MICs between isolates freshly cultured from frozen storage on selective agars with MICs generated from previous antimicrobial susceptibility testing indicated a large degree of concordance. This demonstrated that there appears to be no detrimental influence of agar type in terms of their selectivity on the MICs generated via AST.

Subsequent investigations considered the selectivity of nutrient broths that had been chosen based on their ability to enrich bacterial species of interest for their perceived value in AMR monitoring. EE broth was selected for preferential enrichment of *E. coli*, and AZ broth for enrichment of *Enterococcus* spp. A nutrient broth which was selective for *E. coli* only was not known to be available, and others that were known to select for it also encouraged culture of other bacterial species. Therefore, EE broth, which was described as an enrichment medium for *Enterobacteriaceae,* was selected. AZ broth was described as a detection medium for *Enterococcus* spp. from water samples. Due to the broad description of the selectivity of EE broth and the use of AZ broth for water samples, investigation of their use for enrichment of *E. coli* for the former and enrichment of milk samples for the latter was required.

Investigations demonstrated that EE broth enriched the population of *E. coli* from raw bulk tank milk samples, as demonstrated by increased growth of colonies

morphologically identified as *E. coli* on both selective and non-selective agars following an initial incubation period of 24 hours. Enriched growth of *Enterococcus* spp. colonies following incubation of both single and double strength AZ broths after 24 hours of broth incubation was observed. However, there was unwanted growth of other bacterial species, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* on selective and non-selective agars plated from EE broth, both at 24 and 48 hours of incubation. Similarly, for both AZ broth strengths considered, despite *Enterococcus* spp. colonies being observed most frequently across selective and non-selective agar plates, growth of other bacterial species was found. Differences between single and double strength AZ broths to enrich *Enterococcus* spp. were not considered quantitatively. However, major differences between plates in terms of number of colonies grown were not identified visually.

Both EE and AZ broths were found to enrich the populations of *E. coli* and *Enterococcus* spp. respectively from raw bulk tank milk samples. Therefore, MICs derived direct from broth were largely found to be representative of *E. coli* and *Enterococcus* spp. as confirmed by identification of colonies grown on pure plates as part of the AST by MALDI-TOF MS. However, the presence of other bacterial species identified through the plating processes at the two stages of broth incubation indicate that there is the chance for contamination of a presumably strict measure of *E. coli* and *Enterococcus* spp. MICs.

Broths such as the ones investigated in this work are most commonly used for selective enrichment of environmental samples, after which isolation of bacteria is achieved by use of another selective medium (Saroj et al., 2008). The isolation of morphologically identifiable *E. coli* and *Enterococcus* spp. following selective culture from enrichment broths allowed for comparative determination of MICs of pure isolate culture. This step acted as a validation for the direct broth AST method. Overall high levels of agreement between broth and agar MIC determination methods were observed. Differences in MICs may be attributed to two or more bacterial species being present in the enrichment broth samples used as part of AST. Interestingly, this perceived disadvantage may actually prove to be of value, as it may show the sample

and subsequent MIC measure to be more representative of the highest value of overall resistance.

When analysing comparisons between enrichment broth and agar testing methodologies, antimicrobials with less than 90% agreement were re-evaluated ± one MIC measures. Following this check, a greater number of antimicrobials had ≥ 90% agreement. A target of 90% agreement was set due to standards set for the development of new commercial AST, according to a working group of CLSI (Humphries et al., 2018). The development of numerous approaches for AST has been acknowledged. The working group suggest that any new methods should be compared to a BMD method as a reference, which is described as being a gold standard. Validation of any new methodology is measured by categorical and essential agreement, suggested to be ≥ 90% between the experimental and reference methods. The investigations of direct broth testing benefitted from being carried out via a BMD method, with validations being carried out according to that methodology too. Therefore, the use of this method may prove to be a promising alternative to standard AST.

Additional investigations were carried out to assess the differences between MICs obtained for antimicrobials at two different times of incubation. Broths were incubated for up to 48 hours in an attempt to increase their selectivity for bacteria of interest. Concordance between investigative periods varied across antimicrobials and the media from which testing was carried out. This highlights that time of incubation may have an effect on MICs. However, these differences were investigated on only three out of the eight weekly samples sourced from farms. Therefore, further investigation of potential differences may be warranted to assess these which would better inform an overall enrichment broth AST methodology.

In addition to investigations regarding methodology, this work also represented the opportunity to monitor patterns of MICs for each farm across a period of frequent, weekly sampling. Differences between and within farms were identified and have been described. MICs for select antimicrobials were unchanged in both broth and comparative agar testing, indicating innate or consistently elevated resistances. Small changes across sampling weeks for individual farms were observed. It is difficult

to ascertain whether these changes were related to selection pressures on bacterial populations in the farm environment, due to chance or other influences, or were in fact due to measurement errors associated with the MIC. Consideration of the MIC profiles showed heightened measures for double strength AZ broth when compared to single strength. For example, Farm 5 had MICs corresponding to the highest MIC measure for almost all antimicrobials tested for most weeks sampled. Elsewhere, MIC results were higher than when compared to results obtained from single strength AZ broth. Due to this broth being more concentrated, bacterial populations may have been denser when it came to AST, leading to erroneous results.

5.5 Study limitations

Assessment of enrichment broth selectively was determined by visual analysis of colony morphology of agar plates streaked from broth. Colonies which differed morphologically from *Enterococcus* spp. or *E.* coli were selected for identification, however, some bacterial growth may have been presumed as such when in fact they were not. There were also time limitations which impacted the number of individual colonies to be identified. Additionally, bacterial growth on agar plates was not quantified in terms of colony counts or colony forming units (CFU). Such a procedure may have granted more value to assumptions made regarding broth selectivity which was observed only visually.

A second limitation was that when making comparisons between MICs derived via the enrichment broth method and the agar testing method, results were checked for being ± 1 microdilution. Despite being a well acknowledged error of the MIC, actual differences between the two method's ability to provide an accurate measure may have been discounted by taking this into account.

The reduced number of comparisons that could be made between double strength AZ broth and respective SB agar results due to these comparisons only being made for the final three weeks of samples may have led to differences being missed. This was also applicable to the investigation of the length of broth incubation time on

MICs, as this data was only available for three weeks of sampling compared with eight weeks for the whole sampling period.

5.6 Conclusions

The viability of a novel laboratory based approach to antimicrobial susceptibility testing according to the gold standard of MIC determination via broth microdilution was investigated. Attempts to provide validity through comparisons with standardised approaches, reference with previously made conclusions regarding representativeness of mixed samples, were carried out. Comparisons with these standards indicated high levels of concordance as measured via percentage agreement. Although more work is required to increase the validity of this test, this represents a novel method which could be deployed in the laboratory setting requiring less cost and labour, with the benefit of increased representativeness. The results obtained from investigation of this methodology also allowed for consideration of MIC profiles within and between farms and for differences to be explored. This provides the opportunity to investigate potential associations with AMU to explain these differences. To this end, we consider these associations using AMU records for the six sampling farms in Chapter 6.

Chapter 6; A short term comparison of antimicrobial use and antimicrobial resistance on six dairy farms

6.1 Introduction

The investigation of laboratory methods in Chapter 5 to facilitate routine AMR monitoring on the basis of regular sampling at weekly intervals stimulated an interest in AMU on the selected farms. Although associations between AMU and MIC profiles of sentinel bacteria had been investigated in Chapter 2, the six dairy farms sampled in Chapter 5 were of particular interest since large variations in AMU were known to occur between these farms.

As is widely presented in available literature, higher AMU is generally associated with increased AMR. From the work carried out in Chapter 2, statistically significant positive associations between these were not found across a range of antimicrobial classes considered. It was found that the farms sampled in Chapter 2 had generally lower levels of AMU when compared to mainland UK dairy herds, which may have influenced any potential relationship with AMR.

Consideration of AMU for the six herds sampled in Chapter 5 allowed for further investigation of potential associations with AMR. Where records of AMU in Chapter 2 were sourced from veterinary sales records, records of use outlined in this Chapter were taken from on farm medicine recording, held either electronically or from physical copies. MIC values generated via enrichment broths from the laboratory methods outlined in the previous Chapter were used as the outcome variable for analysis.

The aim of research in this Chapter was to quantify AMU from both high and low usage dairy herds and ascertain whether associations existed between AMU and antimicrobial susceptibilities as measured by MICs.

6.2 Materials and methods

6.2.1 Farm recruitment

As described in Chapter 5 (5.2.1) farms were recruited on the basis of a convenience sample of six farms local to the laboratory with pre-existing commercial and personal contacts. For the purposes of AMU data, medicine records for these farms were easily accessible.

6.2.2 Data export and handling

For five of the six farms, medicine records were held electronically and one farm held physical copies of this data. Electronically held records were filtered (to include only treatments using antimicrobials) and exported as comma separated values (CSV) files to include the following information: treatment date, animal identifier (ear tag number), antimicrobial product used, treatment length (in days), quantity of antimicrobial product used (tubes/mls per day) and animal sex, parity and breed. For the one farm where records were held physically, data were transferred to an Excel spreadsheet in the same format as the data exported electronically.

Following data export, columns were added to recode the information present for each farm. This procedure followed that described in Chapter 2 (2.2.4.1), but is explained again here for convenience. Recoding of records consisted of; a simplified product name/identifier, simplified quantity of product (in ml) and information relating to the active ingredient(s) of each antimicrobial product. To define active ingredient information, a database produced by the VMD (Veterinary Medicines Directorate, 2019) was accessed, with the database being filtered to show products across four antimicrobial categories. The information for each antimicrobial product provided by this database allowed determination of the active substance(s) and its pharmaceutical form (i.e. solution for injection, oral solution or intramammary suspension). This was used to define which active substance(s) constituted the product, and what antimicrobial class it belonged to e.g. amoxicillin was assigned as

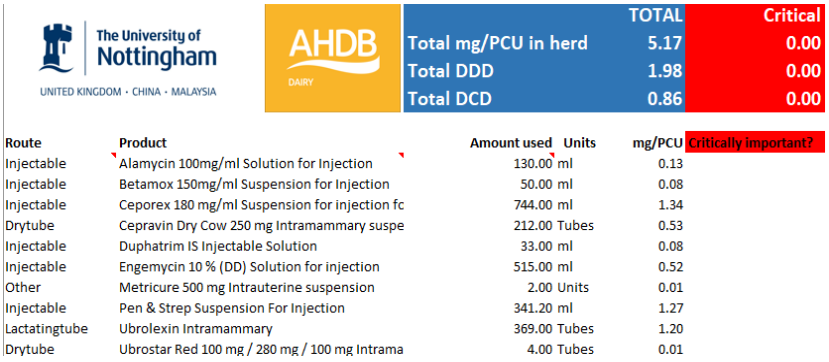
a β-lactam. Where a product contained two or more active ingredients, each individual component of the product was defined in the same manner. Also provided in the VMD database were links to the product data sheets, which indicated the quantity of active ingredient contained per 1ml of solution, or in the case of intramammary suspension, per syringe, in milligrams. These data were used to calculate the total amount of antimicrobial active ingredient that was dispensed/purchased on a given date. For each record entry from the sales data, the total amount of antimicrobial was calculated, in grams of active substance.

6.2.3 Descriptive analyses

For each farm's records, AMU data were also entered into the University of Nottingham/AHDB Dairy AMU (AMU) Calculator (University of Nottingham/AHDB, 2022). This calculator uses data on dairy herd size and total AMU to calculate mg/PCU for the herd as a measure of usage. Additionally, calculations of AMU in terms of total DDD (defined daily dose) and total DCD (defined course dose). Use of critically important antimicrobials is also highlighted within the calculator. Individual antimicrobial products used were identified and categorised in terms of their route of application; injectable, lactating tube, dry tube, footbath or other. The amount of antimicrobial product used across the period covered by the AMU records (January 2021 – March 2022) was entered, from which AMU, measured according to the previously mentioned metrics, was generated. An example of how this was carried out is provided in

Figure 6.1.

Further descriptive analysis of each farm's AMU records were carried out to assess the differences between farm in terms of products, and quantity of products, used. A graphing procedure using the *ggplot2* package in R allowed for a visual representation of the data. From the datasets prepared for each farm, data were transformed to include that relating to date and total antimicrobial (in grams) for each antimicrobial class only, and subsequently plotted quarterly.



	TOTAL	Critical
Total mg/PCU in herd	5.17	0.00
Total DDD	1.98	0.00
Total DCD	0.86	0.00

Route	Product	Amount used	Units	mg/PCU	Critically important?
Injectable	Alamycin 100mg/ml Solution for Injection	130.00	ml	0.13	
Injectable	Betamox 150mg/ml Suspension for Injection	50.00	ml	0.08	
Injectable	Ceporex 180 mg/ml Suspension for injection fc	744.00	ml	1.34	
Drytube	Cepravin Dry Cow 250 mg Intramammary suspe	212.00	Tubes	0.53	
Injectable	Duphatrim IS Injectable Solution	33.00	ml	0.08	
Injectable	Engemycin 10 % (DD) Solution for injection	515.00	ml	0.52	
Other	Metricure 500 mg Intrauterine suspension	2.00	Units	0.01	
Injectable	Pen & Strep Suspension For Injection	341.20	ml	1.27	
Lactatingtube	Ubrolexin Intramammary	369.00	Tubes	1.20	
Drytube	Ubrostar Red 100 mg / 280 mg / 100 mg Intrama	4.00	Tubes	0.01	

Figure 6.1. Example of entry of data into the University of Nottingham/AHDB Dairy AMU (AMU) Calculator using route of application, name of antimicrobial product and amount of product used.

6.2.4 Statistical analysis of associations with minimum inhibitory concentrations

Laboratory data on antimicrobial susceptibilities generated in Chapter 5 from bulk tank milk samples from each of the six study farms was used to investigate associations between MICs and AMU.

As outlined previously in this thesis, MIC data for each farm were used to create an overall 'score' as a measure of antimicrobial susceptibility. As this data had been gathered on a weekly basis, a score was generated for each week. This was achieved by taking the mean of MICs for all antimicrobials tested for each farm for each week, following the rescaling procedure which has been described previously (Chapter 5, 5.2.3.1). Weekly 'scores' were calculated as 'Gram positive' and 'Gram negative'. These were derived from the MICs generated from the enrichment broth methodology from Chapter 5, with results from single strength AZ broth being used as a measure of resistance for Gram-positive and EE broth as a measure of resistance for Gram-negative. These measures would be used as the outcome variables in statistical analysis carried out.

To investigate temporal associations, values for AMU were calculated corresponding to each weekly bulk tank sampling date. Milligrams of active ingredient of antimicrobial product was calculated for: (i) the month prior to the collection of bulk tank samples, and (ii) the week prior to the collection of bulk tank samples. Monthly and weekly measures of AMU were also converted to a measure of this on a mg/PCU basis. Additionally, associations were investigated for individual antimicrobial classes which represented all of each farm's overall use. Antimicrobial classes considered were; aminoglycoside, β-lactam, cephalosporin and macrolide. Although tetracyclines were used across some of the farms, no tetracycline antimicrobials were tested on the Micronaut-Mastitis 3 microdilution plates. Total use of each class was calculated for the year up to the commencement of sampling (mid-January 2022) and until its conclusion (mid-March 2022) and was measured in milligrams. An additional calculation was made for the total use of each antimicrobial class which closely corresponded to the first quarter of 2022 (January 2022 – mid-March) measured in milligrams. Values for all AMU were underwent a log base 10 (log10) transformation due to the large variation in AMU between farms, to normalise the data for modelling.

Statistical analysis of associations was carried out using mixed effects linear regression, due to repeated measurements (samples) being taken from each of the farms over time. This approach was used in Chapter 2 previously (2.2.6) but in brief the models can be described as;

$$Yi = \beta 0 + \beta 1 Xi + \mathrm{Uj} + \mathrm{eij}$$

where Yi represented the dependent variable (weekly mean MIC), $\beta 0$ represented the intercept, $\beta 1$ denoted the coefficient, Xi represented the independent variable (AMU), Uj denoted the separate effects of each mixed effect unit (farm unit) and eij represented the unknown error of the model. Uj and eij were assumed to be normally distributed with mean = 0 and variance Σ_u or Σ_e.

Statistical significance was set using a *t-value*, in which a value of >1.96 or <-1.96 were deemed significant (equivalent to P<0.05).

6.3 Results

6.3.1 Study population characteristics

As of the commencement of bulk tank sampling in January 2022, the herd size of farms constituting the study population ranged from 40 to 360 adult dairy cows, with a mean herd size of 233.

6.3.2 Descriptive results of antimicrobial use

Analysis of veterinary sales records highlighted a high degree of variation between farms in terms of the quantities of antimicrobials used and the number of different antimicrobial classes used on each farm. These classes were; aminoglycoside, amphenicol, β-lactam, cephalosporin, macrolide, sulfonamide, trimethoprim and tetracycline. Use of classes varied across the six farms; Farm 1 used only 3 out of 8 classes recorded, while 3 farms (Farms 3, 5 and 6) used 7 out of 8 the aforementioned classes. All six farms used aminoglycosides, β-lactams and cephalosporins. Table 6.1 provides a descriptive outline of the proportion of overall AMU for each farm as a percentage of overall use. Aminoglycosides, β-lactams and cephalosporins comprised the majority of use across all farms.

Table 6.1~~6.1~~. An outline of descriptive statistics of the variation of use between classes of antimicrobials, expressed as percentages of overall use, across all farms.

Farm ID	Antimicrobial Classes (% mass of overall use)						
	Aminoglycoside	Amphenicol	β-lactam	Cephalosporin	Macrolide	Sulfonamide/ Trimethoprim	Tetracycline
1	43.4	-	32.1	24.5	-	-	-
2	42.1	-	44.5	10.3	-	-	3.2
3	44.4	-	48.9	3.2	2.1	1.16	0.17
4	22.4	-	13.8	48.4	-	1.4	13.9
5	42.9	1	35.5	13	-	1.7	5.8
6	44.3	-	36.7	0.9	2.4	10.5	5.1

Calculations of AMU using the three metrics of mg/PCU, DDD and DCD highlighted variation between farms. An overview of the values of these metrics are provided in Table 6.2. Mean AMU for mg/PCU, DDD and DCD was found to be 12.98, 3.35 and 1.28 respectively. Farm 5 was the farm that used a critically important antimicrobial, Excenel Flow, an injectable product, containing ceftiofur (third generation cephalosporin).

Table 6.2~~6.2~~. An outline of descriptive measures of AMU calculated for all farms using all AMU data from January 2021 – March 2022.

Farm ID	Metrics of Antimicrobial Use					
	mg/PCU	Critical mg/PCU	DDD	Critical DDD	DCD	Critical DCD
1	2.18	0	0.75	0	0.23	0
2	10	0	1.34	0	0.73	0
3	29.73	0	8.74	0	3.27	0
4	5.17	0	1.98	0	0.86	0
5	26.38	0.02	6.83	0.02	2.45	0
6	4.44	0	0.47	0	0.14	0

Figures 6.2 – 6.7~~3~~ provide a visual representation of the patterns of AMU between farms during the study period.

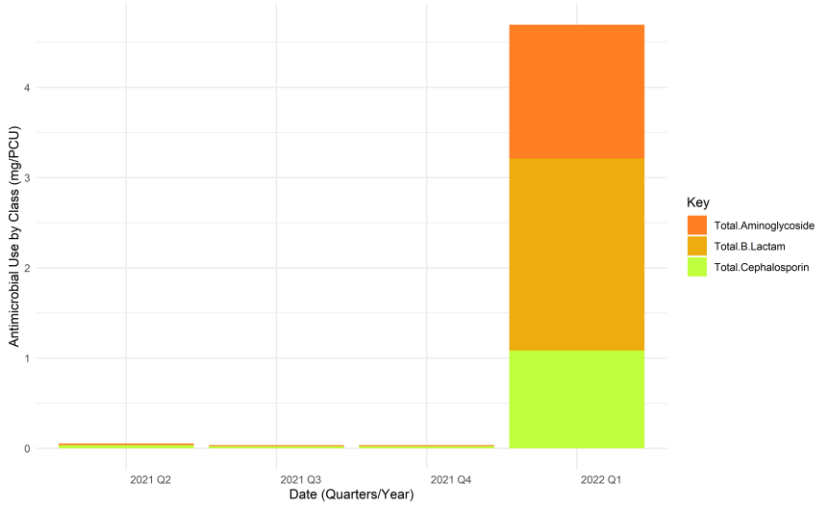


Figure 6.2. Graphical illustration of AMU for Farm 1 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

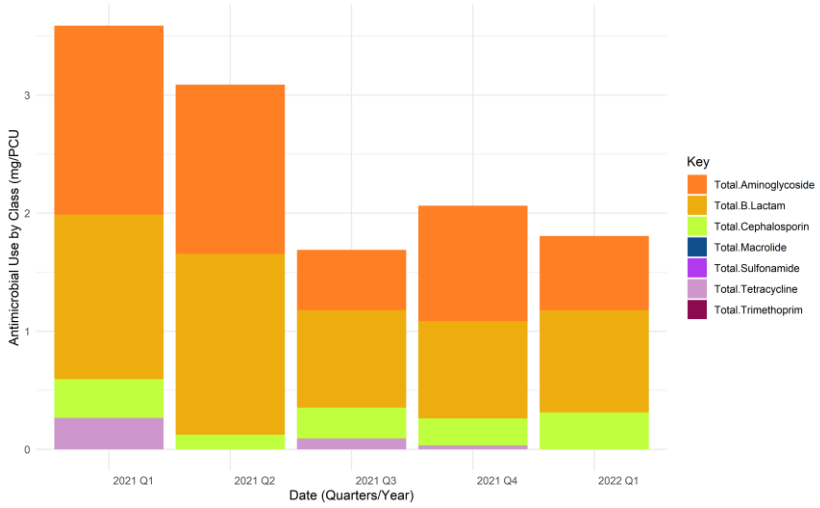


Figure 6.3. Graphical illustration of AMU for Farm 2 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

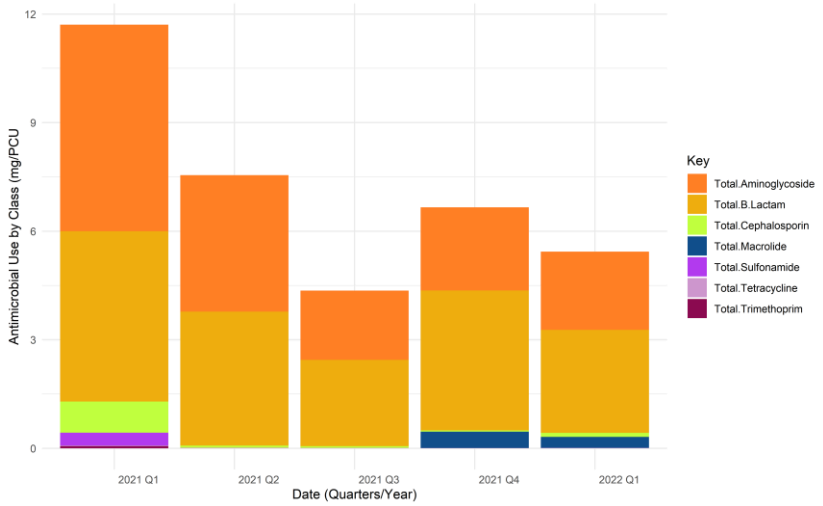


Figure 6.4. Graphical illustration of AMU for Farm 3 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

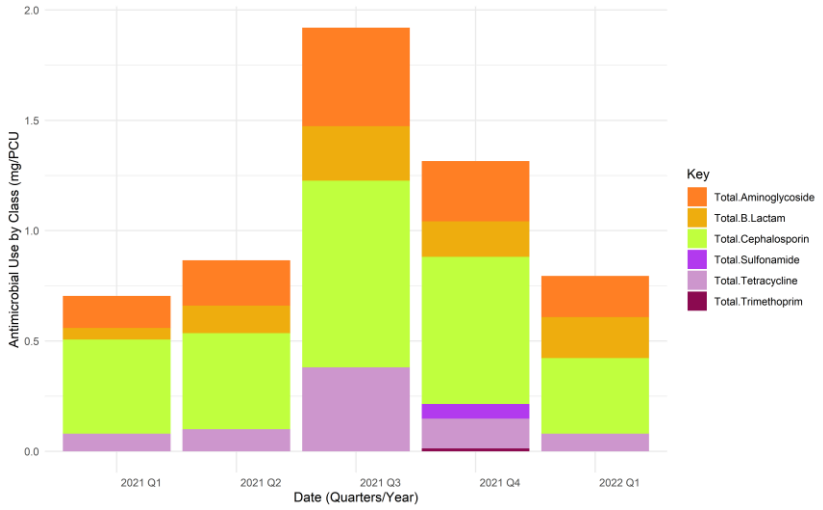


Figure 6.5. Graphical illustration of AMU for Farm 4 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

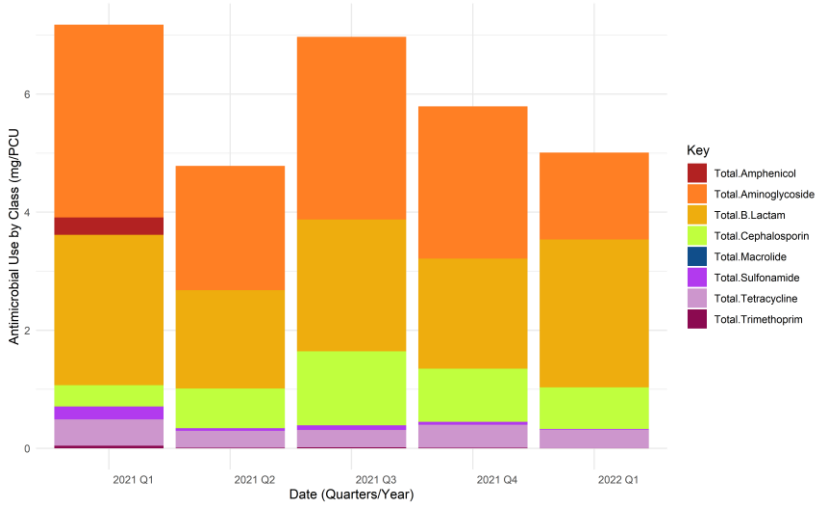


Figure 6.6. Graphical illustration of AMU for Farm 5 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

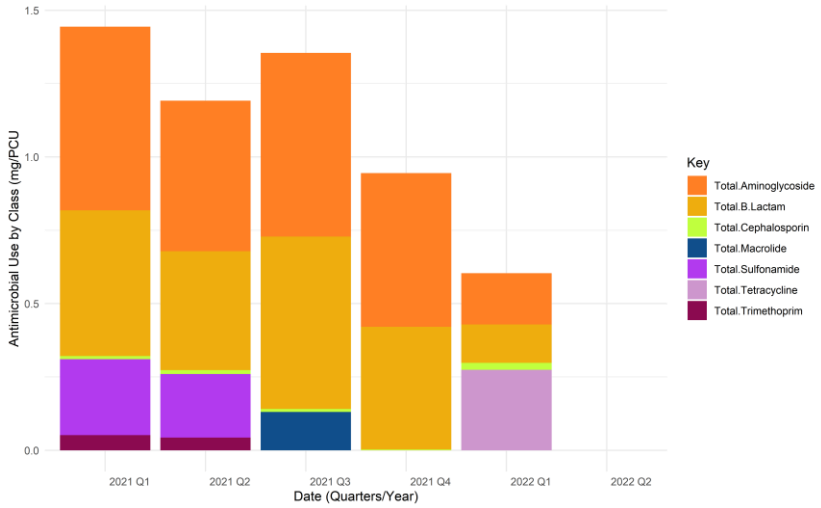


Figure 6.7. Graphical illustration of AMU for Farm 6 between January 2021 and March 2022, presented in terms of use by antimicrobial class, measured by mg/PCU, on a quarterly basis.

6.3.3 Statistical analysis of associations

Commented [DM11]: As per correction feedback, models were re-run without farm 1. Results did not change massively. Log scaling was employed to account for differences in scale between farms to account for the huge variation between farm 1 and the others.

Statistical analysis of associations between measures of AMU and weekly MIC means via mixed effects linear regression modelling for Gram positive and Gram negative are presented in Table 6.3 and Table 6.4 respectively. Table 6.3 shows these data for all AMU use regardless of antimicrobial class, while Table 6.4 provides an overview of data for measures of AMU for individual classes.

Table 6.3~~6.3~~. An overview of mixed effects linear regression model results showing associations between measures of AMU (total use and by mg/PCU metric) and Gram positive and Gram negative weekly mean MICs. Green shading indicates a statistically significant relationship (P<0.05), while red shading indicates ~~the converse.~~no statistically significant relationship present.

Measure of AMU	Gram-positive	Standard Error	Gram-negative	Standard Error
mg total use for previous month	0.081	0.208	-1.961	0.256
mg/PCU for previous month	-0.54	0.517	-0.947	0.753
mg total use for previous week	-0.232	0.047	-0.887	0.064
mg/PCU for previous week	-1.292	0.71	-1.777	0.912

Table 6.4~~6.4~~. An overview of mixed effects linear regression model results showing associations between measures of total AMU for individual antimicrobial classes and Gram positive and Gram negative weekly mean MICs means. Green shading indicates a statistically significant relationship (<0.05) while red shading indicates ~~the converse.~~ no statistically significant relationship present.

Measure of AMU for individual antimicrobial classes	Gram-positive	Standard Error	Gram-negative	Standard Error
mg total use of aminoglycosides for previous year	1.149	0.137	-1.838	0.202
mg total use of aminoglycosides for first quarter	0.172	0.222	-2.24	0.263
mg total use of β-lactam for previous year	1.016	0.143	-2.053	0.189
mg total use of β-lactam for first quarter	-0.077	0.192	-2.363	0.222
mg total use of cephalosporin for previous year	-0.619	0.157	-1.172	0.254
mg total use of cephalosporin for first quarter	-1.354	0.205	-1.226	0.368
mg total use of macrolide for previous year	-1.327	0.159	0.308	0.161
mg total use of macrolide for first quarter	-1.855	0.187	-0.121	0.225

6.4 Discussion

The selection pressures placed on bacterial populations by antimicrobials has been argued to be the most significant driver behind the emergence of AMR (Holmes et al., 2016). Within agriculture, and specifically in the dairy industry, AMU has been found to influence AMR in bacterial populations; higher levels of use have been found to be associated with higher levels of resistance (Catry et al., 2016; Saini et al., 2012).

In this Chapter, the associations between AMU and antimicrobial susceptibilities as measured by MICs were investigated from a convenience sample of six dairy herds.

These herds had been selected based on prior knowledge of their AMU patterns, with herds varying from generally lower use to higher usage.

AMU in terms of overall usage, when measured by mg/PCU varied greatly between farms, with the two highest farms both being greater than ten times higher than the lowest usage farm. The mean mg/PCU for these herds was found to be 12.98, compared with a mean of 8.3 mg/PCU for the study farms investigated in Chapter 2. As with those farms, aminoglycosides, β-lactams and cephalosporins were found to be the most frequently used antimicrobials.

Despite such a range within the study group, higher levels of AMU were not associated with corresponding higher MICs in this study. Where statistically significant relationships were found, the effects of these were always negative, indicating that higher usage farms had lower antimicrobial susceptibilities as expressed via a mean MIC. These findings were exclusively for the Gram-negative score, with no statistically significant relationships found for the Gram-positive score.

A study by Firth et al., (2022) explored the relationship between AMU and the presence of antimicrobial resistant bacteria on dairy farms in Austria. The authors collected environmental faecal and dust samples for the isolation of *E. coli* and methicillin-resistant *Staphylococcus aureus* respectively. AMU was measured according to DDDvet/cow/year. Farms were grouped according to relative AMU; high and low levels of use. The authors found no statistically significant difference between high and low AMU groups associated with the presence of AMR bacteria. Although this study may not be directly reflective of the work presented in the current Chapter, it does suggest that relationships between AMU and AMR are not always directly positively associated. In contrast, a longitudinal study over four years investigating these associations in Belgian livestock (Callens et al., 2018) found that decreasing levels of AMU were associated with a decrease in AMR of commensal *E. coli* isolates. The authors suggested that AMU reduction policies would have "beneficial effects on overall resistance levels". However, dairy herds were not included in this study which featured different methodology regarding collation of AMU data for the livestock species considered. Despite this, the study is concurrent

with widely published literature suggesting positive associations between AMU and AMR, which, the results presented in this Chapter, do not allude to.

However, the surprising results presented in this Chapter may be explained due to the nature of the data used in analysis. Despite MIC data showing small levels of variation within farms across the eight weeks of sampling as well as between farms, the overall variability was perceived to be generally low. Additionally, due to AMU data being attributed to weekly bulk tank sampling, repeated measures of the same AMU amount were used in analysis. Together, these two factors may have led to a spurious determination of the relationships between the AMU and MICs considered here, rather than a biological reasoning. However, further consideration may help to determine a true reasoning for these findings.

6.5 Study limitations

This study only considered a small sample size of six farms, smaller yet again than the sixteen farms studied in Chapter 2. Inclusion of greater number of farms with varying levels of AMU may have been beneficial for investigation.

Weekly mean MIC scores which were used as the outcome variables as part of statistical analysis were deduced from enrichment broths rather than individually cultured isolates. Although this may constitute a more representative sample of the bulk tank milk sample, this methodology makes it hard to compare and contrast with other studies considering the associations investigated here. This is due to the fact that standard antimicrobial susceptibility testing is carried out according to the culture and selection of individual bacterial isolates.

From Chapter 3, it was concluded that areas of farm management were found to be associated with differences in MICs between farms. Such investigations were not undertaken for farms featured in this work. Therefore, consideration of potential differences may have helped to further inform the findings presented here.

6.6. Conclusions

Relationships between AMU and AMR were further investigated using on farm medicine records and the latter in terms of laboratory methodology outlined in the previous Chapter which utilised enrichment broths in the determination of MICs. In conclusion, it has been established that, despite large differences between farms in terms of their AMU profiles, statistically significant positive relationships between AMU and AMR could not be found. The findings from this work may help to inform future studies on the relationships between AMU and AMR and bring attention to findings which do not fit with the general acceptance of the positive relationships which have been widely found.

Chapter 7; General discussion

7.1 The relationship between antimicrobial use and resistance on dairy farms

The research presented in this thesis sought to further our understanding of the dynamics of AMU and AMR interactions in the dairy farm environment. The microbiology of bulk tank samples, which have been claimed to be a representative sample of the dairy cow environment (Berge et al., 2007), provided a medium through which to culture and isolate sentinel bacterial species. These bacteria have been suggested as having value in terms of monitoring due to their ubiquity in the farm environment and an ability to acquire and disseminate AMR related genes. Quantification of AMU, obtained through veterinary sales data and via on farm medicine use records allowed for relationships between use and resistance to be explored. Data relating to farm management practices allowed us to investigate the dynamics of AMR on dairy farms further by exploring non-AMU related factors which may influence levels of resistance on farm.

Chapter 1 outlined a review of available literature and provided a context and rationale for the work constituting this PhD project. The emergence and dissemination of AMR and the risks posed to human health as a result were considered, alongside modes of antimicrobial action and subsequent resistances that bacterial species have obtained. The scale of AMU in agriculture, having been identified as a driver of AMR globally was examined. The subsequent need to reduce AMU and to monitor AMR in agriculture provided justification for this research, with specific focus on the dairy industry.

From this rationale, a number of overarching investigative aims were explored. Three main objectives of study contributed to the overall findings of this research;

(i) Investigation of associations between AMU and AMR via longitudinal analysis
(ii) Identification of associations between non-AMU related factors (farm management practices) and levels of on farm resistance

(iii) Investigation of the viability of novel laboratory approaches to be employed as part of a routine AMR monitoring programme.

7.2 Overview of chapters and conclusions

Implementation of a longitudinal study of the interactions between AMU and AMR, as presented in Chapter 2, allowed for the initial aim of investigation of such associations to be examined. Pre-existing commercial contacts and archived bulk tank milk samples allowed for a study of long terms trends of AMU and levels of AMR as measured via MICs to be carried out. The sixteen study farms offered a unique opportunity to study an isolated, closed island population with minimal external influences. A study period encapsulating five years of AMU obtained via veterinary sales data and six years of MIC data for sentinel bacteria species allowed for an extensive set of data to be considered. Investigation of the dynamics between AMU and AMR were revisited as part of Chapter 6, with the benefit of sample farms being actively selected for based on already established assumptions of AMU.

Outcomes of analyses from the data presented in these Chapters were indeed surprising. Despite the breadth of data collected as part of Chapter 2 and the range of AMU of farms selectively recruited in Chapter 6, few statistically significant relationships were found. This led to the conclusion, that, although not found here, AMU is still a significant driver of AMR. There exists complex dynamics when considering AMR with simultaneous investigation of other potential influences being warranted. To this end, consideration of farm management practices was explored.

Drawing on conclusions from Chapter 2 and literature suggesting the influence of farm management systems, for example, organic vs conventional farming (Murphy et al., 2018), a rationale for Chapter 3 was established. Here, an extensive investigation of farm management practices was conducted in order to address the second objective of this overall research. Recruitment of farms from Chapter 2 allowed for questionnaires to be carried out in face to face visits with farmers to capture data relating to practices across a range of farm management areas. Due to the small sample size (n=16), farm management data was supplemented with

questionnaire responses and MICs of sentinel bacteria from previous work (Bradley et al., 2018). Methods regarding bacteriology and antimicrobial susceptibility testing were not identical between studies since the two were independent and carried out at different times, however the principles of data collection and sample handling were the same for both studies. The employment of a robust modelling method, the elastic net featuring a bootstrapping procedure for inference, allowed for associations between farm management practices and corresponding increased or decreased MICs across farms to be identified.

Exploratory investigations for the development of a laboratory method for the monitoring of AMR from bulk tank milk samples were made as part of Chapters 4 and 5. The concept of a 'mixed' MIC, drawing on previous investigations based on *P. aeruginosa* infections in cystic fibrosis patients (Morlin et al., 1994; Van Horn, 1993) was developed. Findings suggested a more representative MIC measure could be obtained from multiple bacterial isolates. From this conclusion, an AST procedure was outlined and validated in Chapter 5.

The viability of a novel laboratory approach to antimicrobial susceptibility testing according to the gold standard of MIC determination via broth microdilution was investigated. Attempts to provide validity through comparisons with standardised approaches, reference with previously made conclusions regarding representativeness of mixed samples, were carried out. Comparisons with these standards indicated high levels of concordance between methods. Although additional work could be done to increase the validity of this test, this represents a novel method which could be deployed in the laboratory setting requiring less cost and labour, with the benefit of increased representativeness. The results obtained from investigation of this methodology also allowed for evaluation of MIC profiles within and between farms and for differences to be explored. This gave the opportunity to investigate potential associations with AMU to explain these differences. To this end, we considered these associations using AMU records for the six sampling farms in Chapter 6.

7.2 Notable findings

Throughout the research and subsequent analyses detailed and presented across the Chapters of this thesis, a number of specific areas of interest arose.

Research carried out as part of Chapter 2 represented a unique consideration of an entire population, rather than a select subsample of a wider population. Added value was brought to this study group through its isolation from external interactions with other livestock and environmental influences, as well as the length of time explored as part of this longitudinal study.

Lower levels of AMU were found in this study group compared to a convenience sample of mainland UK herds (Hyde et al., 2017). The breed in the study herds presented in Chapter 2 were exclusively Jersey. Differences in disease tolerance and ~~wellness~~ health status between Jersey and Holstein/Friesian breeds have been investigated (Bannerman et al., 2008; Gonzalez-Peña et al., 2020) with the Jersey breed having been noted for increased disease tolerance towards mastitis (Washburn et al., 2002). Treatment for mastitis has been shown to be the primary use of antimicrobials in the dairy industry, with levels of mastitis potentially explaining some of the difference in AMU between study populations. Additionally, the implementation and maintenance of the closed herd system being implemented in the island population recruited in Chapter 2 may also provide an explanation for lower AMU (because of a decreased risk of introduction of disease).

The most notable finding from Chapter 2 was that despite a long time series being investigated with a wealth of data available, wide ranging associations between AMU and trends in MICs as a measure of resistance were not found. An association between aminoglycoside use and corresponding MICs in *Enterococcus* spp. for gentamicin was identified. The significance of this finding is however unclear, due to the negative association being found. *Enterococcus* spp. have been shown to exhibit moderate to high-level intrinsic aminoglycoside resistances due low cell wall permeability and aminoglycoside modifying enzymes. (Hollenbeck and Rice, 2012). Therefore, the intrinsic resistances which exist may account for this unclear result. From consideration of AMU across this thesis, aminoglycosides have been found to

be one of the most commonly used antimicrobial classes on dairy farms, most often found in combination with β-lactams. The role of Enterococci as potential reservoirs of AMR related genes may facilitate the dissemination of resistances to other bacterial species via horizontal gene transfer (von Wintersdorff et al., 2016). For this reason, identification of these interactions may hold importance for future research of AMR in the context of dairy.

Similarly in Chapter 6, where statistically significant relationships were found, associations between AMU and MICs were negative. Such associations have been identified in previous work (Saini et al., 2013), with the authors describing this as biologically improbable. Rather than having a biological basis, these results may be explained by measurement errors. Sample populations in Chapters 2 and 6 were both relatively small, with limited variability identified in terms of MIC profiles and AMU.

Findings from Chapter 3 relating to farm management practices constituted novel research into an area of agricultural AMR that receives less attention than that directed at AMU. Previously published literature has acknowledged the differences in AMR between organic and conventional farming systems, but this has usually had a focus on differences in AMU between systems. However, wider investigation of individual areas of farm management and routines has rarely been considered. Investigations carried out represented the opportunity to identify non-AMU related AMR influences between farms. The regularised regression procedure employed was relatively novel in its use of veterinary medicine when outlined by Lima *et al.*, (2020) and provided a robust procedure for analysis of the wide datasets which constituted this chapter. Addition of a second dataset (n=125) to the smaller dataset initially considered as part of the investigation of farm management practices added emphasis to the outcomes which suggest the importance of these influences.

Although associations were identified, the causal nature of these remains unknown. As a result, these findings may help to direct attention and further work to the wider farm environment and its management to provide more comprehensive examination of the dynamics of AMR on dairy farms. One of the most notable associations found was that of slurry storage. Slurry has been acknowledged as representing a significant reservoir of resistant bacteria and genes conferring these resistances. Baker *et al.*,

(2022) recently found that continuous throughput of slurry into storage maintained a resistance equilibrium, with fresh populations of resistant bacteria replacing those at the end of their life cycle. Possible reasoning behind the modelling outcomes identified have been previously discussed. However, indirect effects associated with AMU may provide some causality. For example, use of antimicrobial based materials as part of bedding and housing management may reflect higher AMU in an attempt to control mastitis in the herd.

Finally, the findings from Chapters 4 and 5 exploring possible laboratory methodology for the monitoring of sentinel bacterial population of bulk tank milk samples represent a key finding from the research carried out. Given the scale of the problem which AMR poses, accurate and reliable antimicrobial susceptibility testing according to stringent standards should be adhered to. Therefore, novel testing regimens need to be assessed against broth microdilution, which has been determined as the gold standard of antimicrobial susceptibility testing. As has been described and discussed, the methodology developed had a series of validation steps which were themselves informed using comparisons with a standard AST procedure via BMD.

The new methodology could undoubtedly be improved with additional research quantification as has been acknowledged in Chapter 5. It does however show promise as a monitoring method with the potential for application in the laboratory. Currently, monitoring of AMR across the UK dairy industry is primarily conducted on a voluntary, passive basis and does not constitute a concerted programme of methodical surveillance. The clinical surveillance programme collects samples from carcasses and samples submitted to and analysed by government laboratories for pathogen screening; AST is subsequently carried out on pathogens identified. Results of this passive testing programme are compiled and published by the Veterinary Medicines Directorate (VMD) in the Veterinary Antimicrobial Resistance Surveillance (VARSS) report (Bennani et al., 2021). This therefore highlights a gap which could potentially be filled by the laboratory broth methodology to monitor AMR trends on a national basis and provides a rationale for its employment.

7.3 Potential future work

The outcomes of this research could be improved by greater representativeness in study samples. Across the Chapters presented in this thesis, sample sizes were relatively small. Although investigations of AMU and AMR were longitudinal in nature and captured a significant amount of data, which improves on many previous cross-sectional studies, it is uncertain how representative our study herds were of the wider dairy industry. Herds recruited in Chapter 2 could not be argued to be representative of the UK dairy herd due to the geographic isolation, nor could the herds sampled as part of Chapters 5 or 6 due to their limited geographical range in addition to the small sample size. Therefore, future work examining the situation of AMR in the UK dairy industry should encapsulate a greater number of sample farms.

The results and conclusions drawn from Chapter 3 regarding the uncertainty in causal associations between farm management practices and differences in MICs warrant further investigation. Randomised control trials could be carried out to establish a biological basis for such findings. Further microbiological investigation of the farm environment could be carried out to establish causality for the differences observed for bedding and housing management as well as the associations found relating to the milking parlour. Establishment of the mechanisms behind these associations may present opportunities for further intervention in addition to reductions in AMU to limit the emergence of new resistances.

Further work could be carried out with regards to the broth methodology developed and outlined. The use of other nutrient broths could be investigated and results of their enrichment could be quantified to better inform decisions regarding their use.

Finally, monitoring of AMR in the context of the dairy industry could be expanded to include major pathogens responsible for disease. For example, AMR monitoring of those involved in intramammary infections such as *Streptococcus uberis* and *Staphylococcus aureus*, due to these infections being a primary reason for AMU on dairy farms.

7.4 Conclusion

The research presented in this thesis adds further value to the current understandings of the dynamics of AMR in the dairy industry. Research primarily focused on the associations between AMU and AMR, whilst also exploring the influences of the dairy farm as a whole through the consideration of management practices. AMR was measured via MICs, derived from a broth microdilution method, widely regarded as a gold standard.

The findings of this research contribute to already published literature regarding longitudinal studies of AMU and AMR, with the length of time studied and the uniqueness of the study population considered in Chapter 2 enriching these outcomes. Although a wide range of AMU and AMR data were considered in Chapter 2, there were a lack of statistically significant relationships existing across the data, and where an association was found between aminoglycoside use and MICs for gentamicin in *Enterococcus* spp., this was negative. Reduced variability in MIC profiles as well as lower levels of AMU may have contributed to these findings, in addition to the existence of intrinsic aminoglycoside resistance harboured by *Enterococcus* spp. Similarly biologically implausible findings were found as part of AMU investigations in Chapter 6. However, these findings helped to draw attention to the potential influence of other contributions to AMR at the farm level. Findings presented in Chapter 3 outline a novel investigation of non-AMU factors. A range of areas of farm management were identified as being associated with higher or lower MICs across farms, in addition to AMU influences. However, the causal nature of these associations was unclear and could only be postulated upon. However, these associations represent the opportunity for further investigation to clarify their nature and represent the potential for future interventions to limit AMR emergence in the dairy industry.

This research has also established a novel monitoring method which could be employed to compliment national AMU surveillance data. Consideration of *Enterococcus* spp. and *E. coli* as sentinel bacteria for their ubiquity in the dairy farm

environment and their role as a reservoir for AMR related genes holds further value. Monitoring resistances in these bacteria may better inform wider surveillance.

As the UK dairy industry and the wider agricultural sector works towards reducing overall AMU in an effort to combat AMR and the risk to human health via the food chain, continued monitoring to inform guidelines will be important. To this end, the findings of the research outlined in this thesis help to inform ways in which this can be achieved via a broader approach to the dairy farm environment and enhanced monitoring via a convenient sampling and testing methodology.

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Appendix – Chapter 2

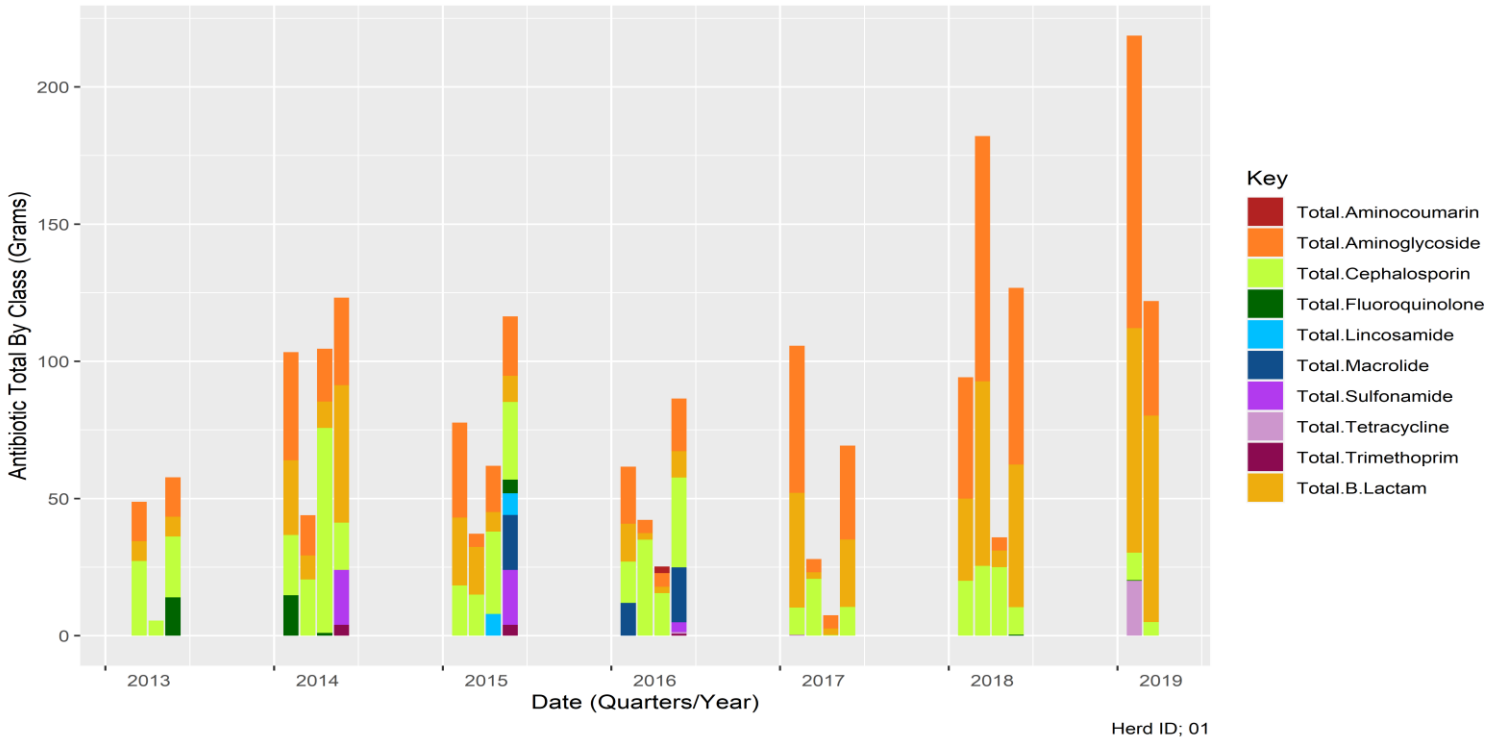


Figure A2.1. Graphical illustration of AMU for Farm 01 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

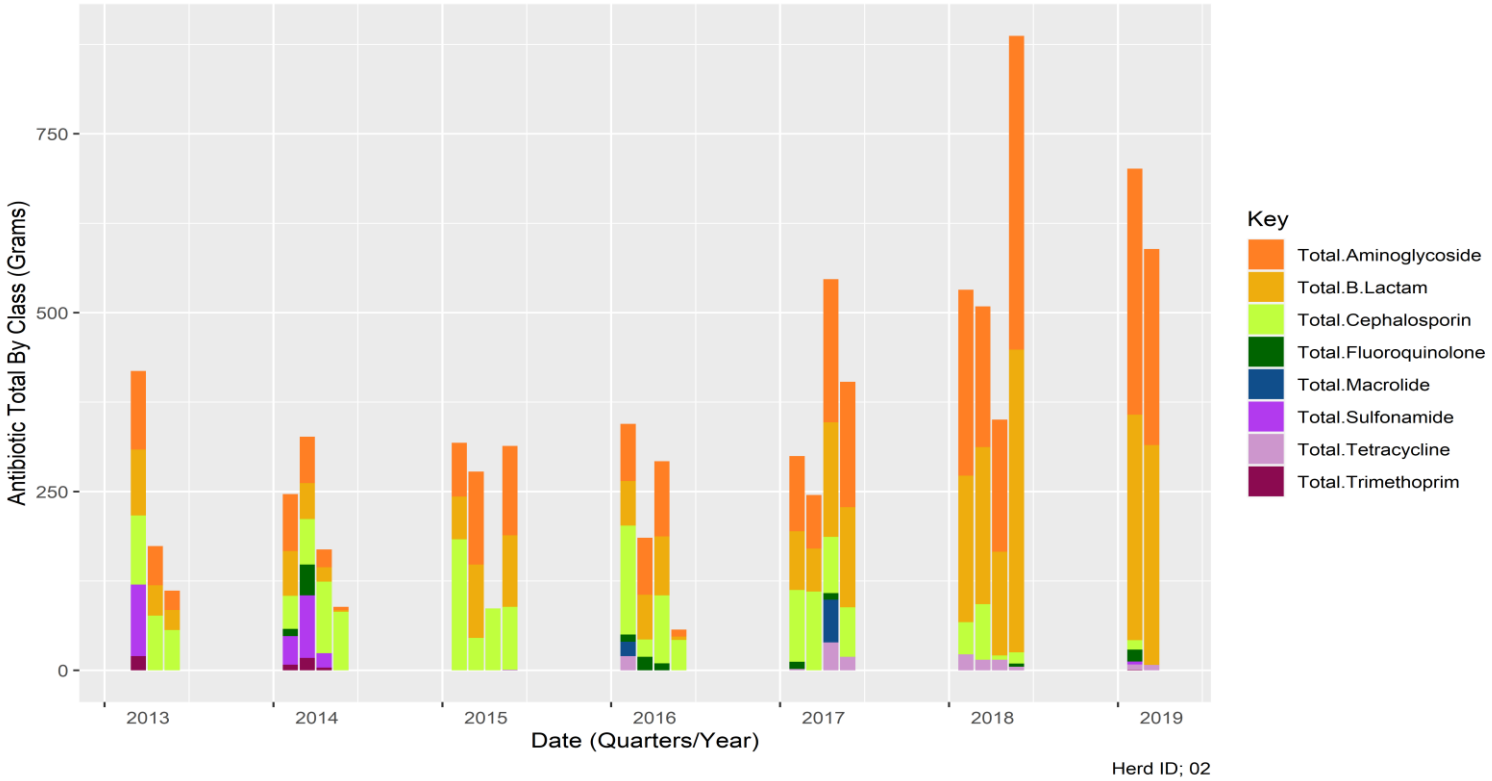
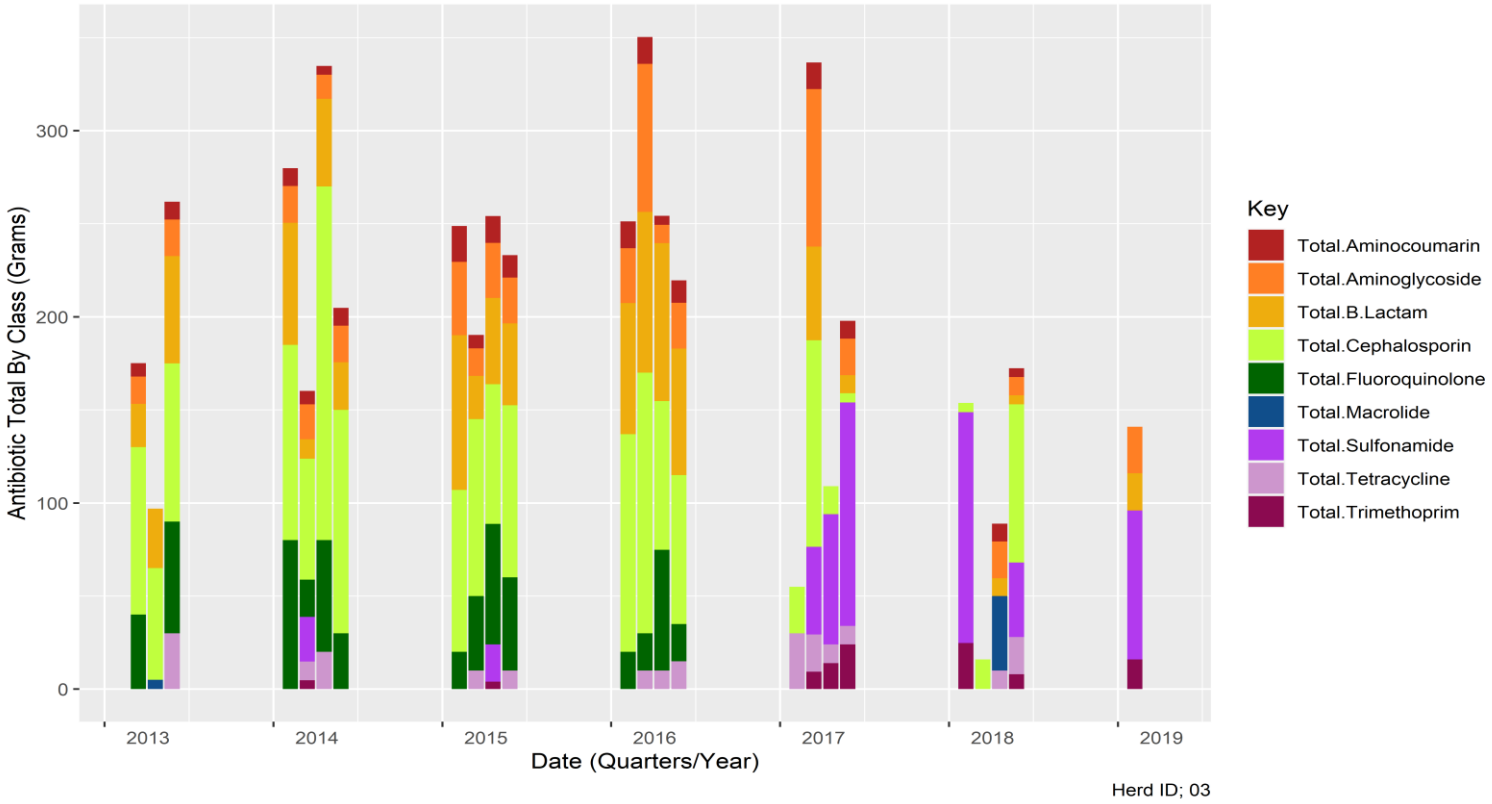


Figure A2.2. Graphical illustration of AMU for Farm 02 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year



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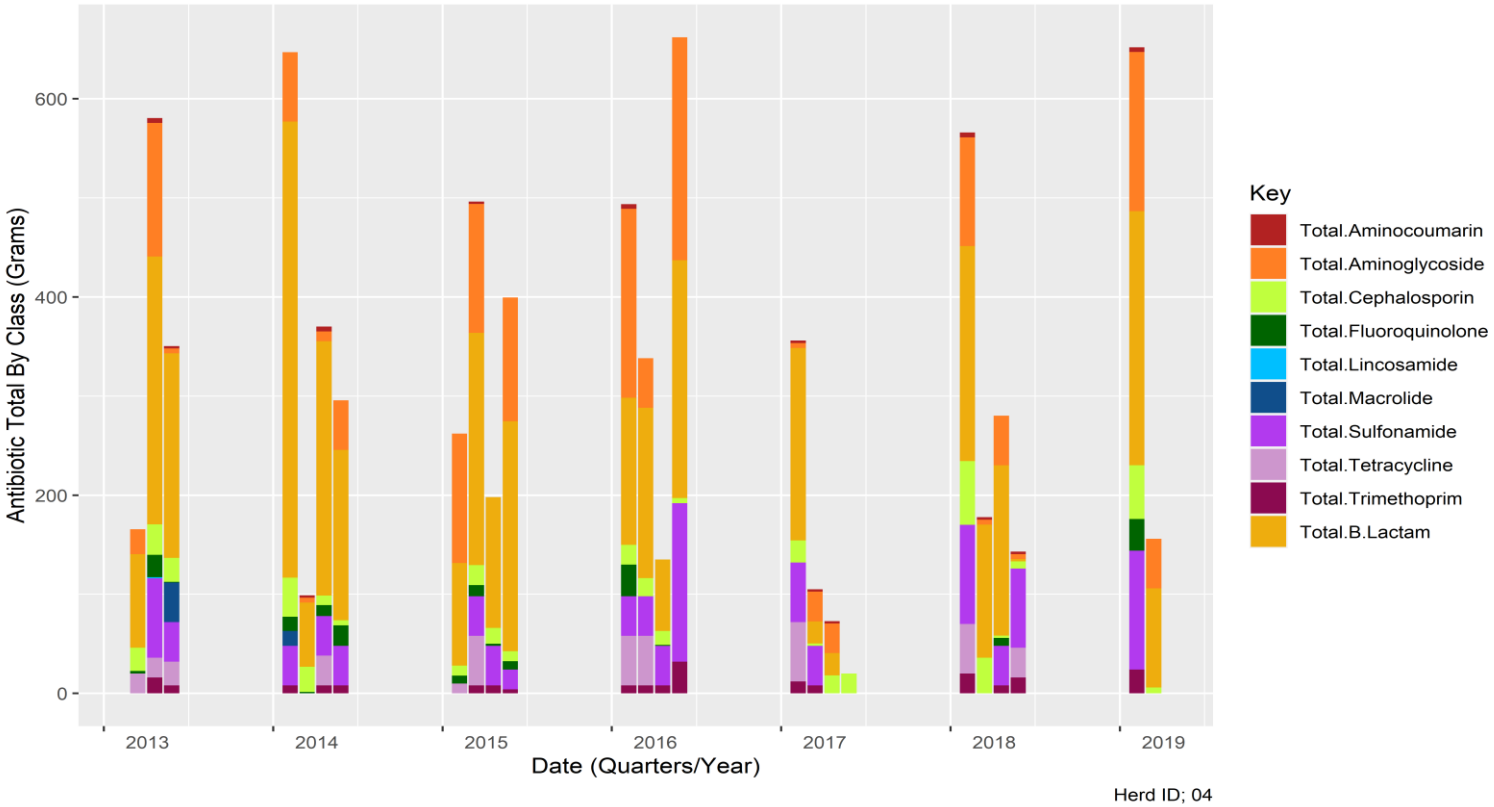

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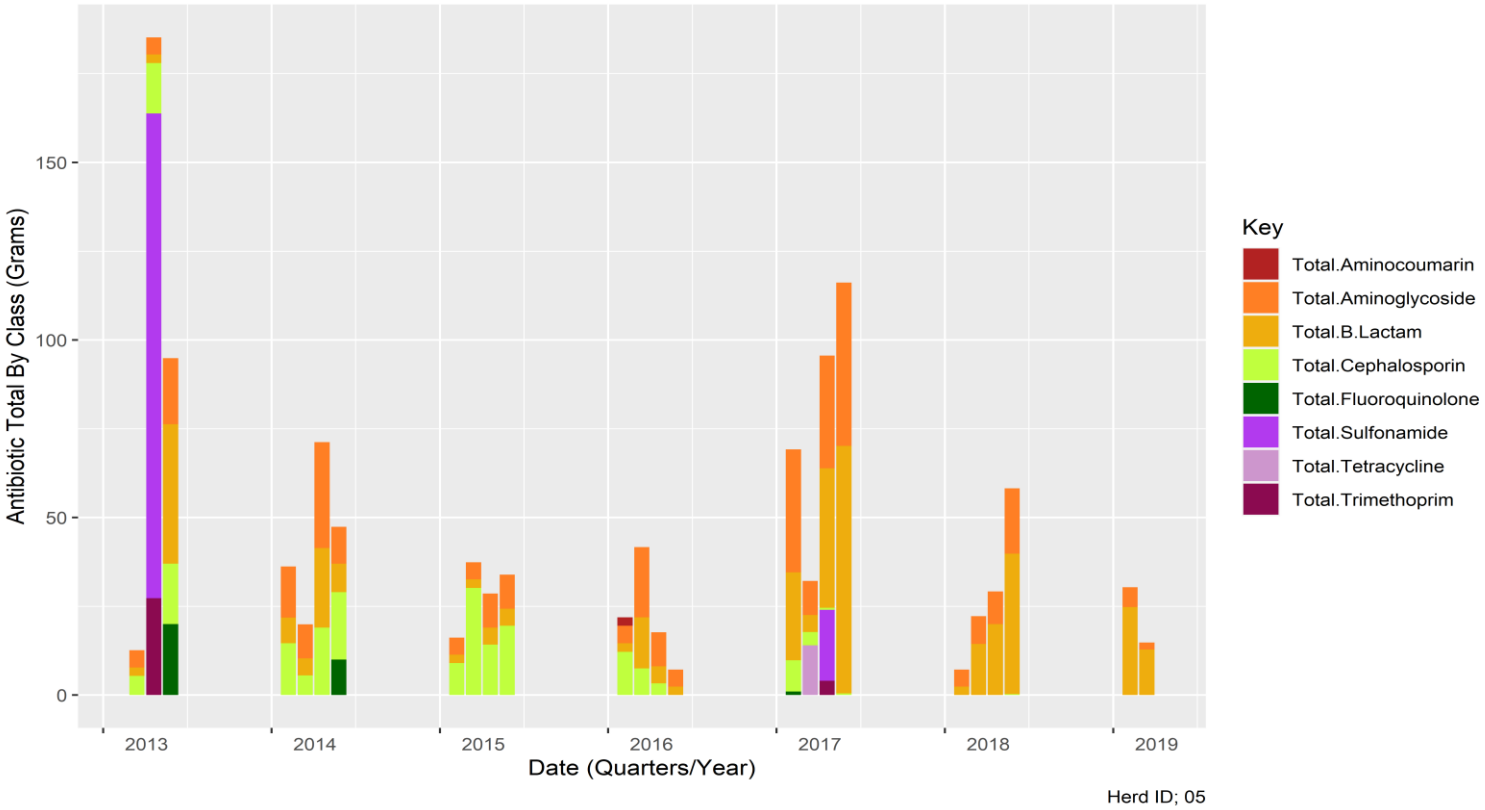

Figure A2.5. Graphical illustration of AMU for Farm 05 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

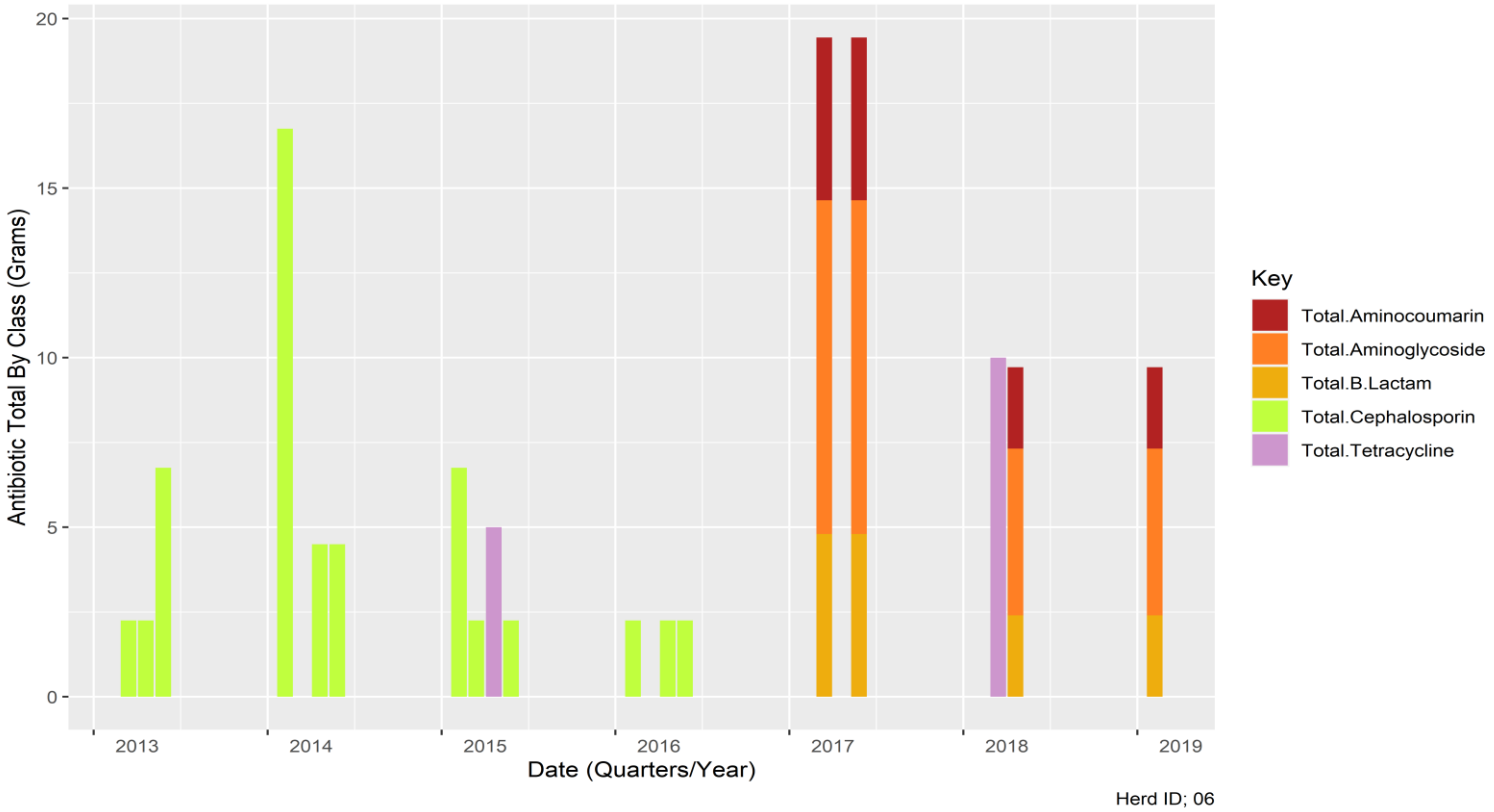

Figure A2.6. Graphical illustration of AMU for Farm 06 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

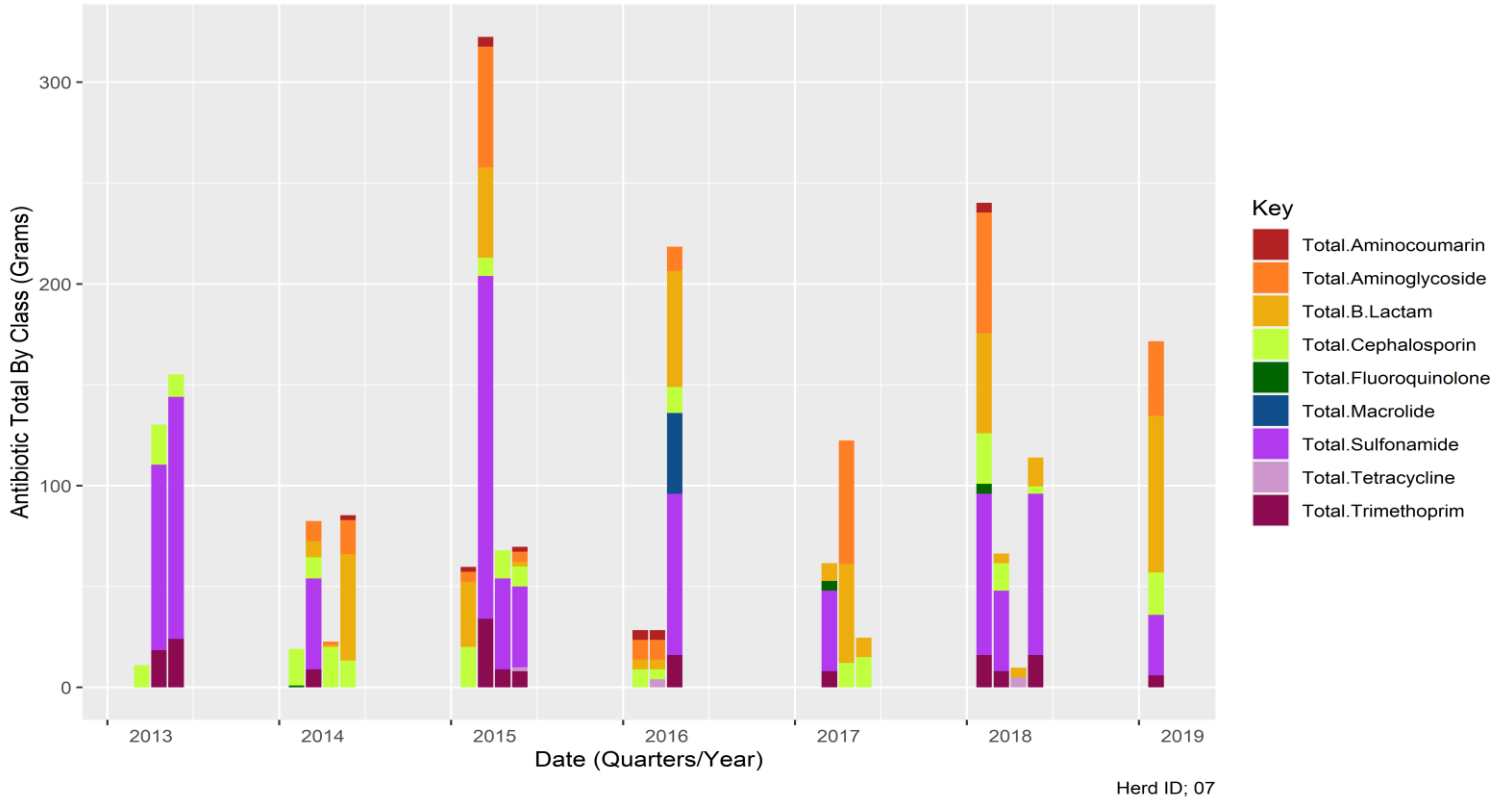

Figure A2.7. Graphical illustration of AMU for Farm 07 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

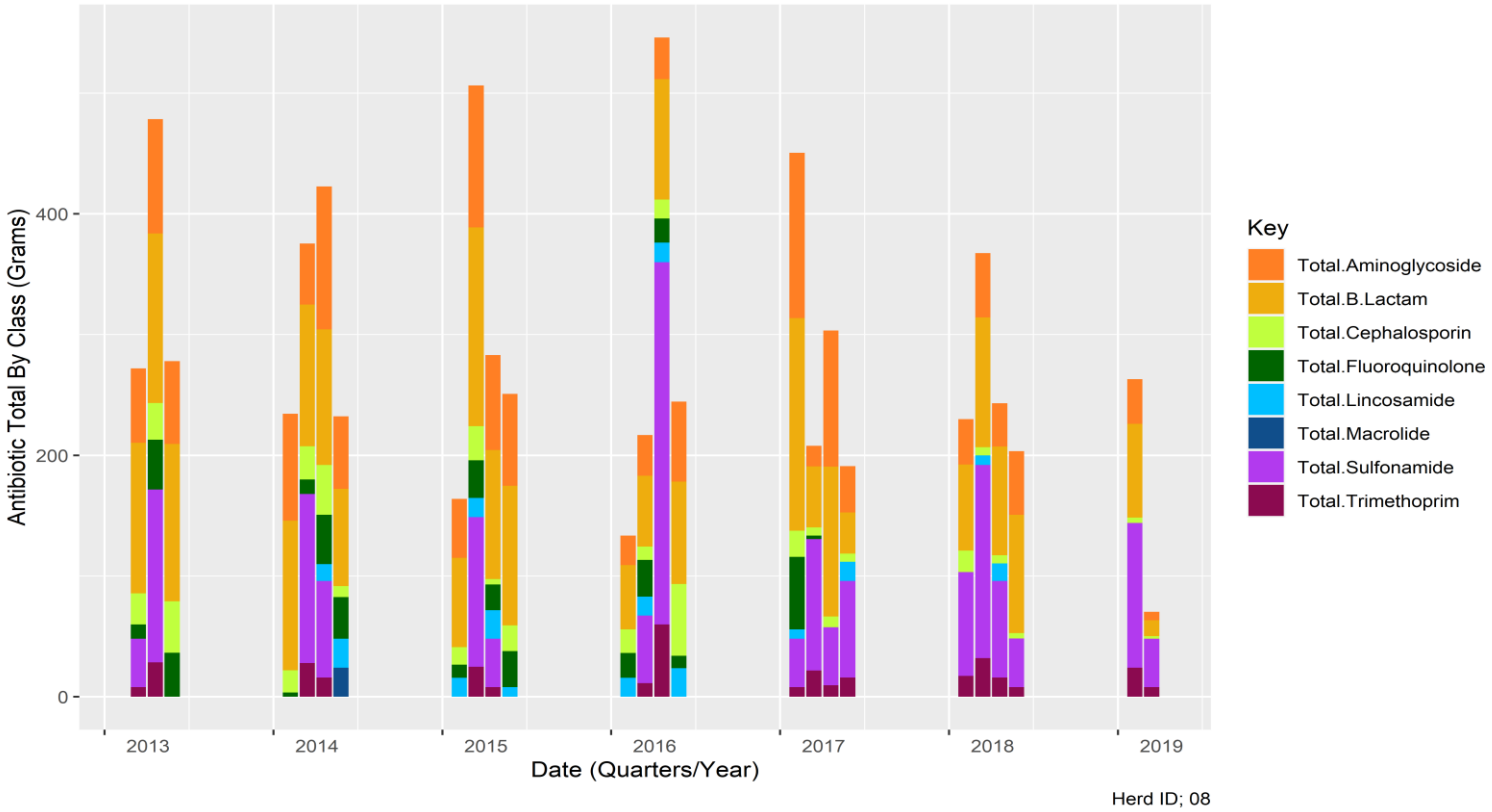

Figure A2.8. Graphical illustration of AMU for Farm 08 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

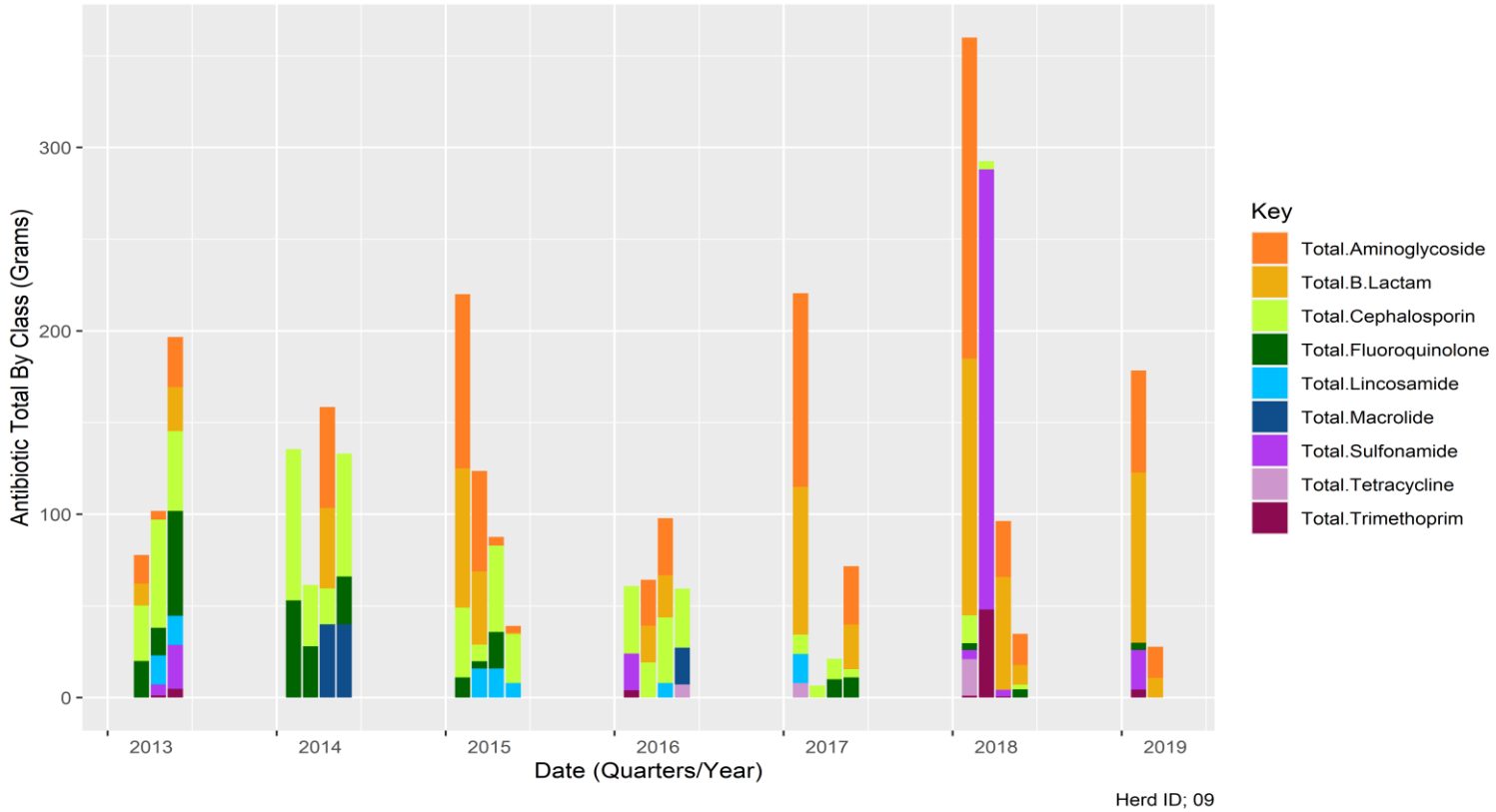

Figure A2.9. Graphical illustration of AMU for Farm 09 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

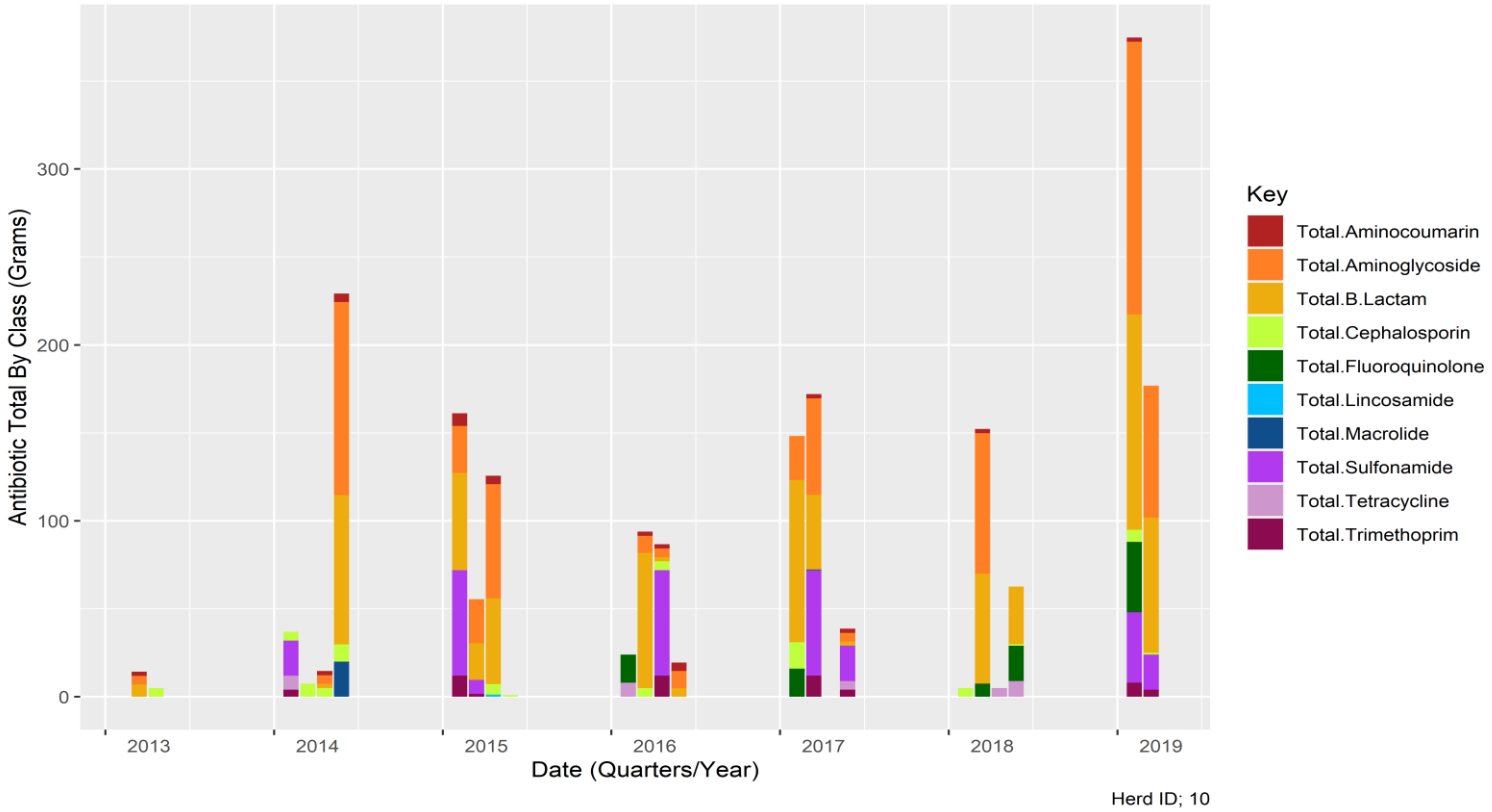

Figure A2.10. Graphical illustration of AMU for Farm 10 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

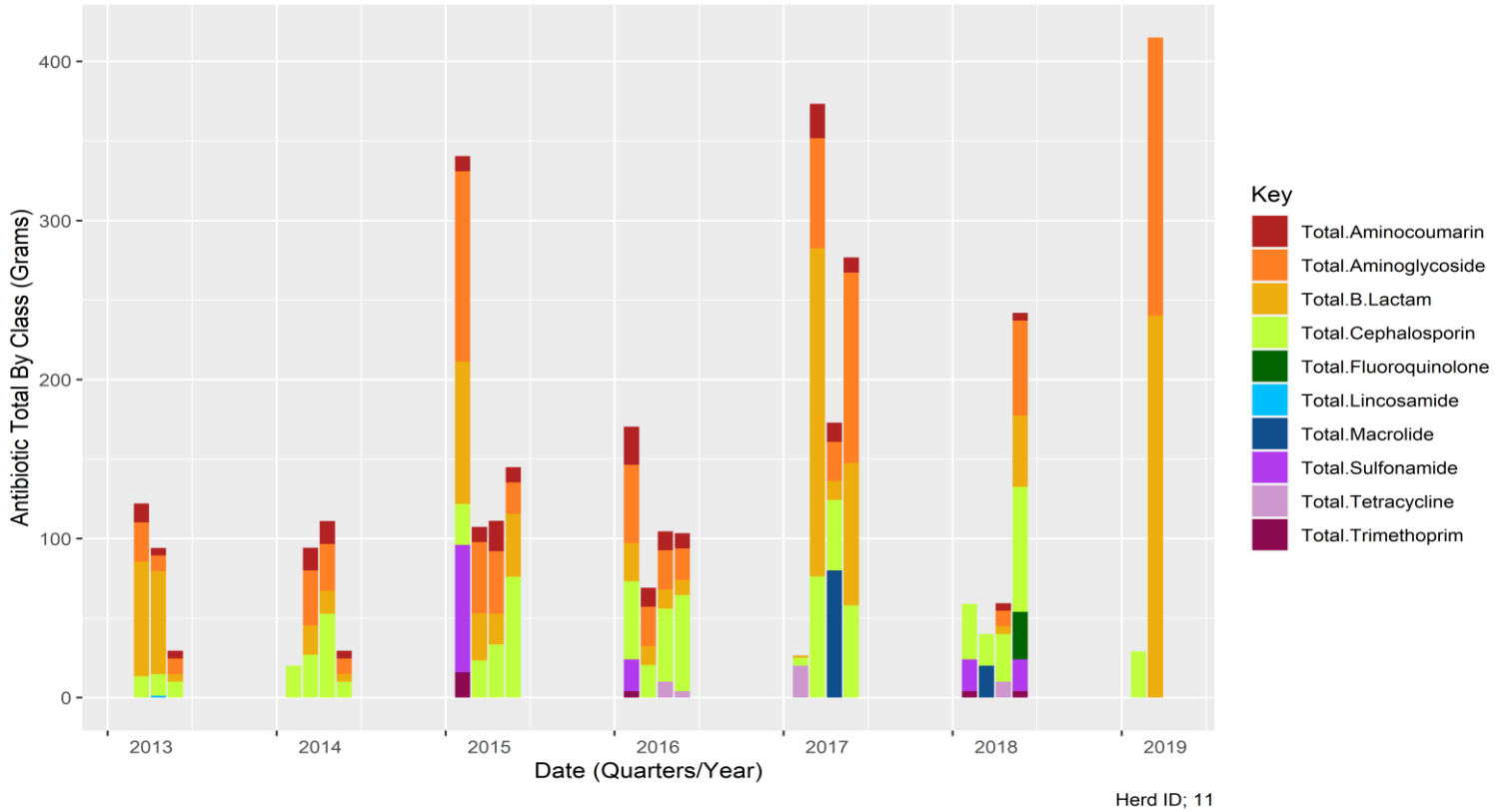

Figure A2.11. Graphical illustration of AMU for Farm 11 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

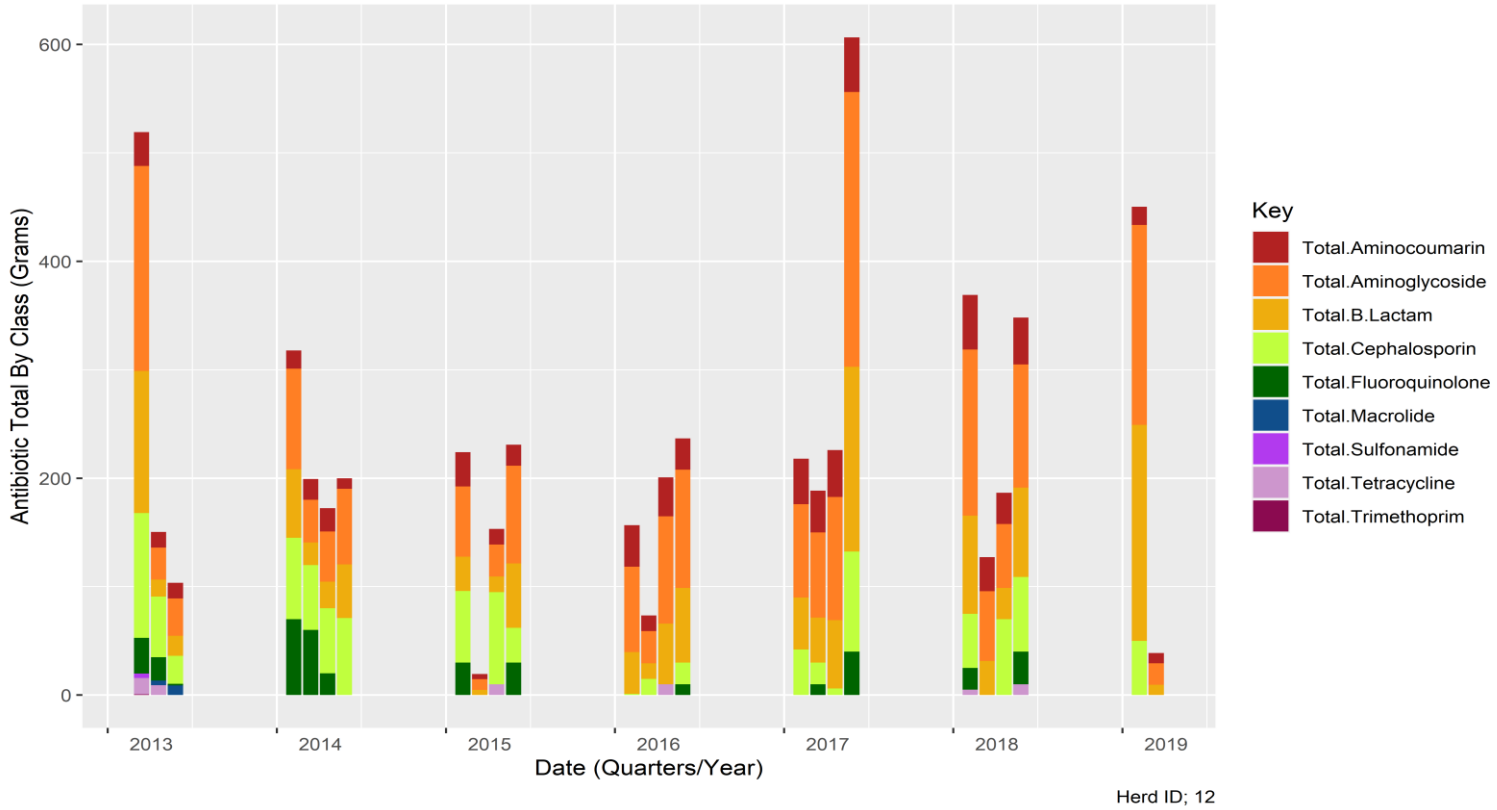

Figure A2.12. Graphical illustration of AMU for Farm 12 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

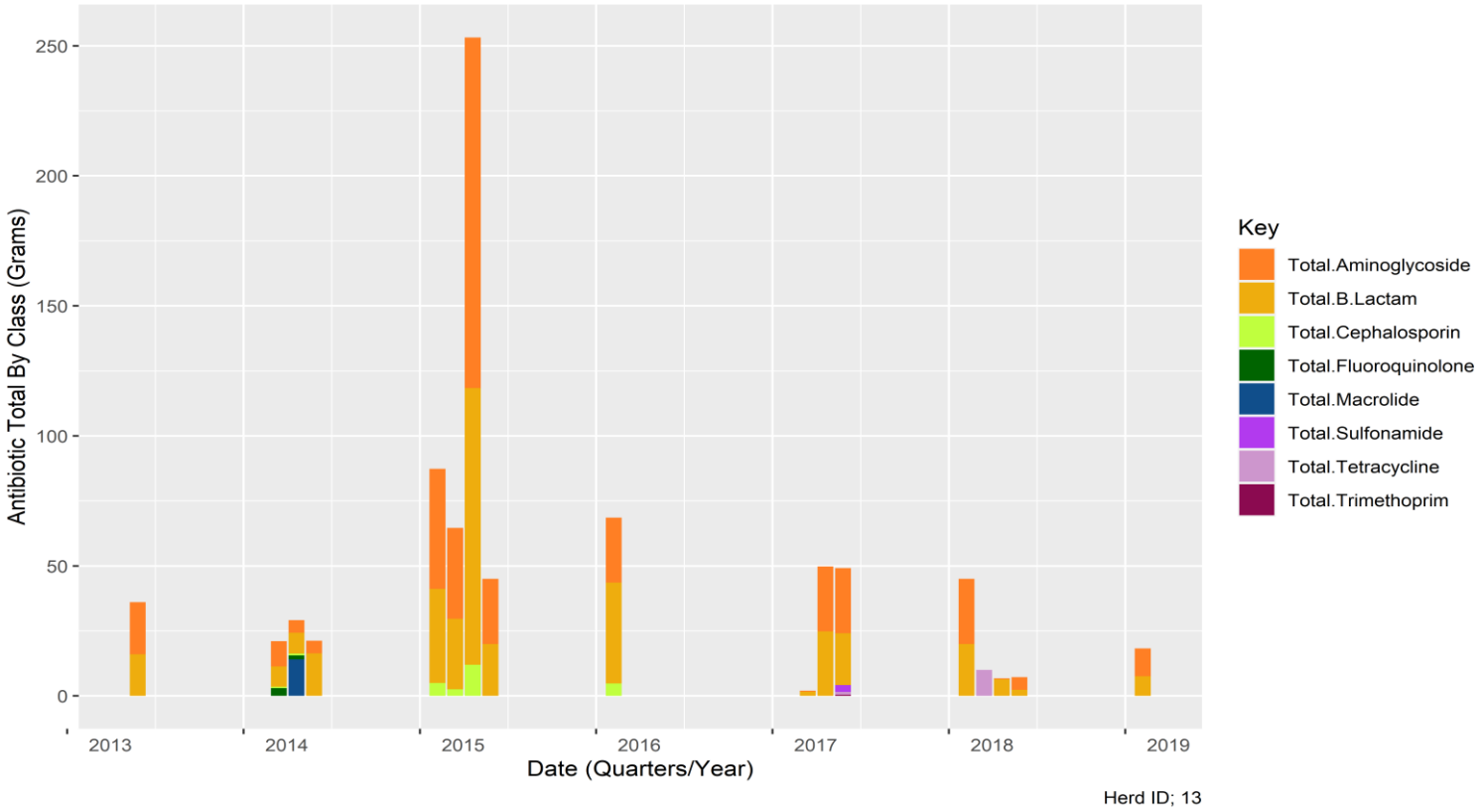

Figure A2.13. Graphical illustration of AMU for Farm 13 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

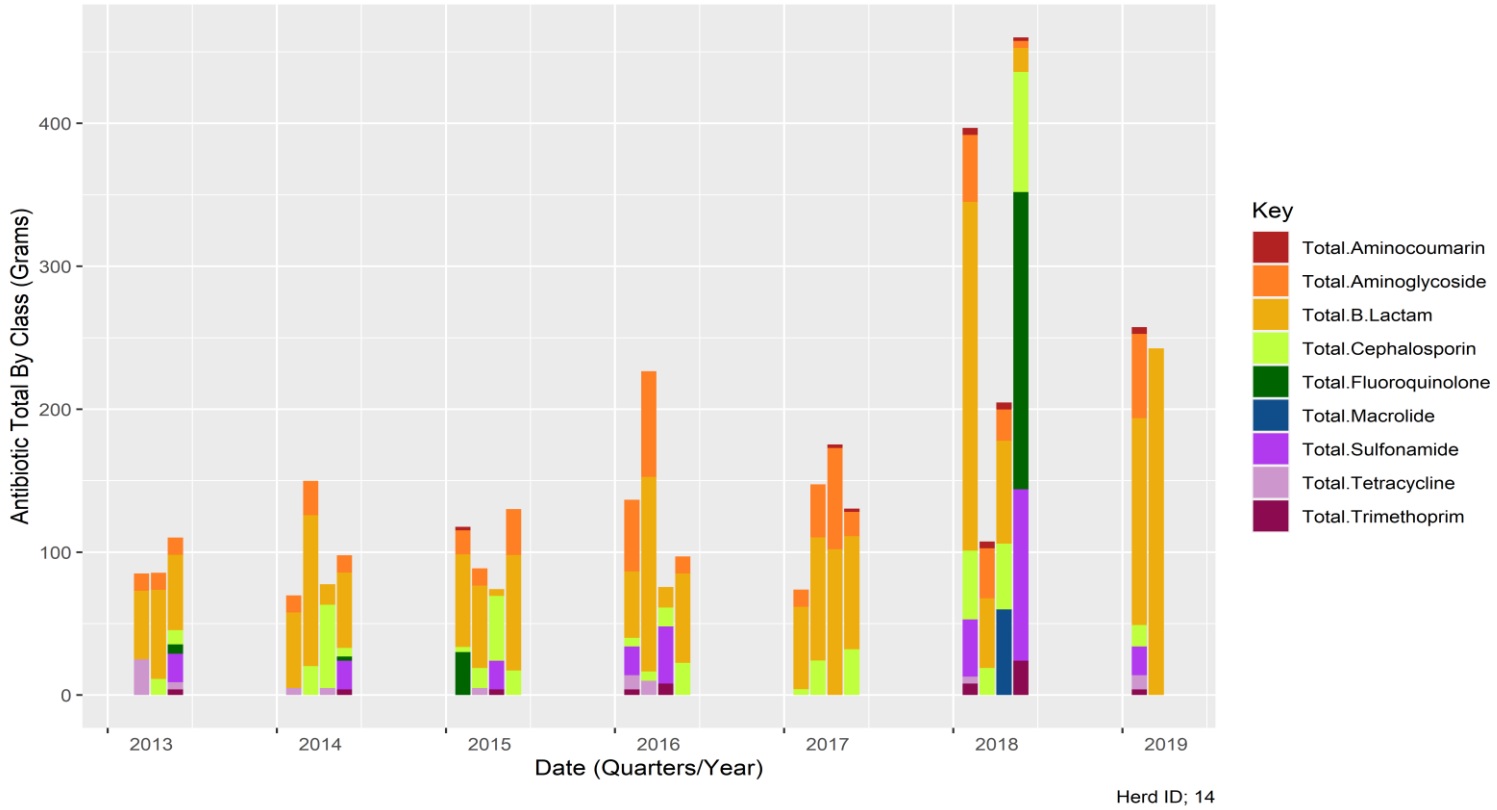

Figure A2.14. Graphical illustration of AMU for Farm 14 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

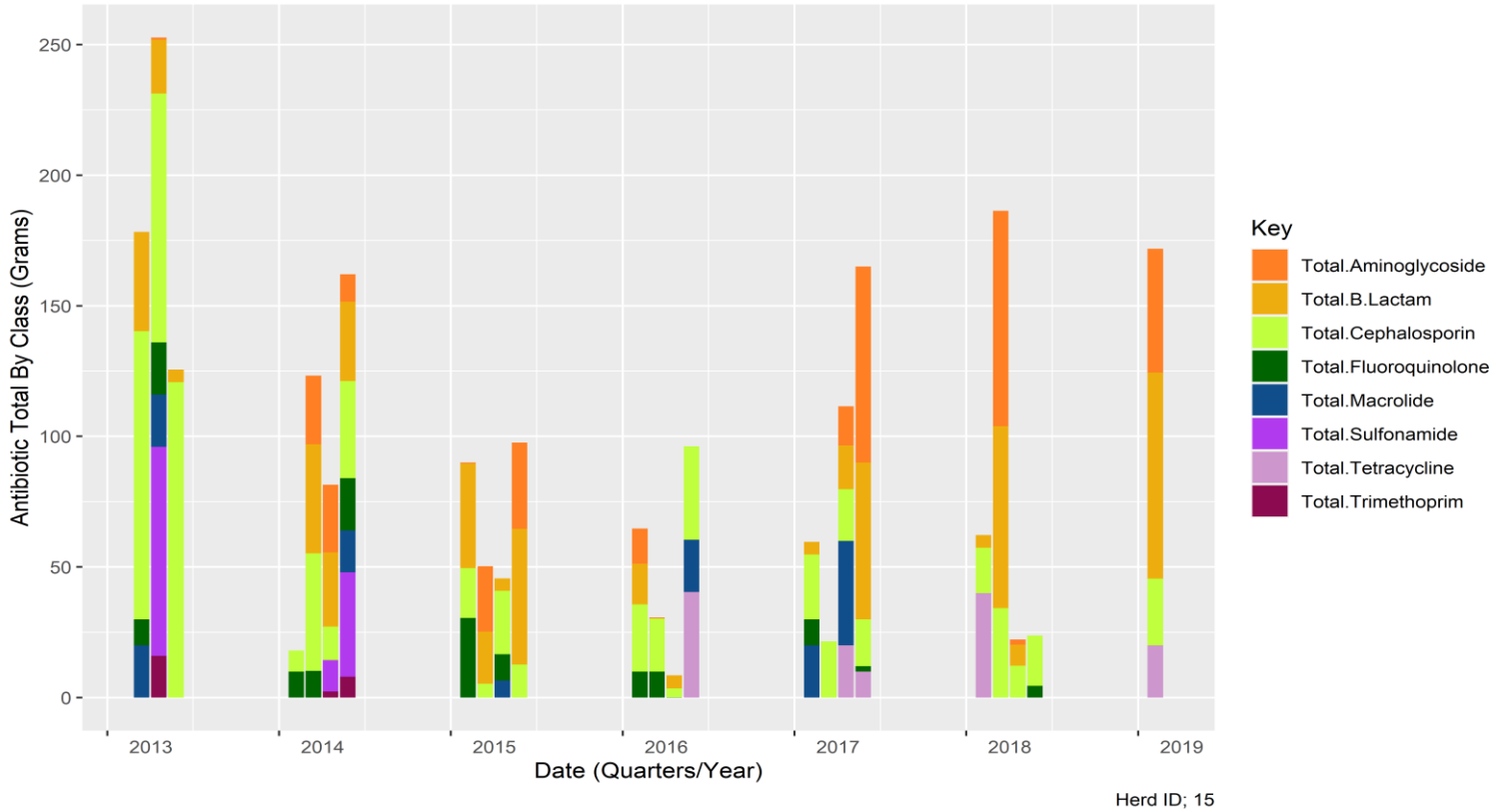

Figure A2.15. Graphical illustration of AMU for Farm 15 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

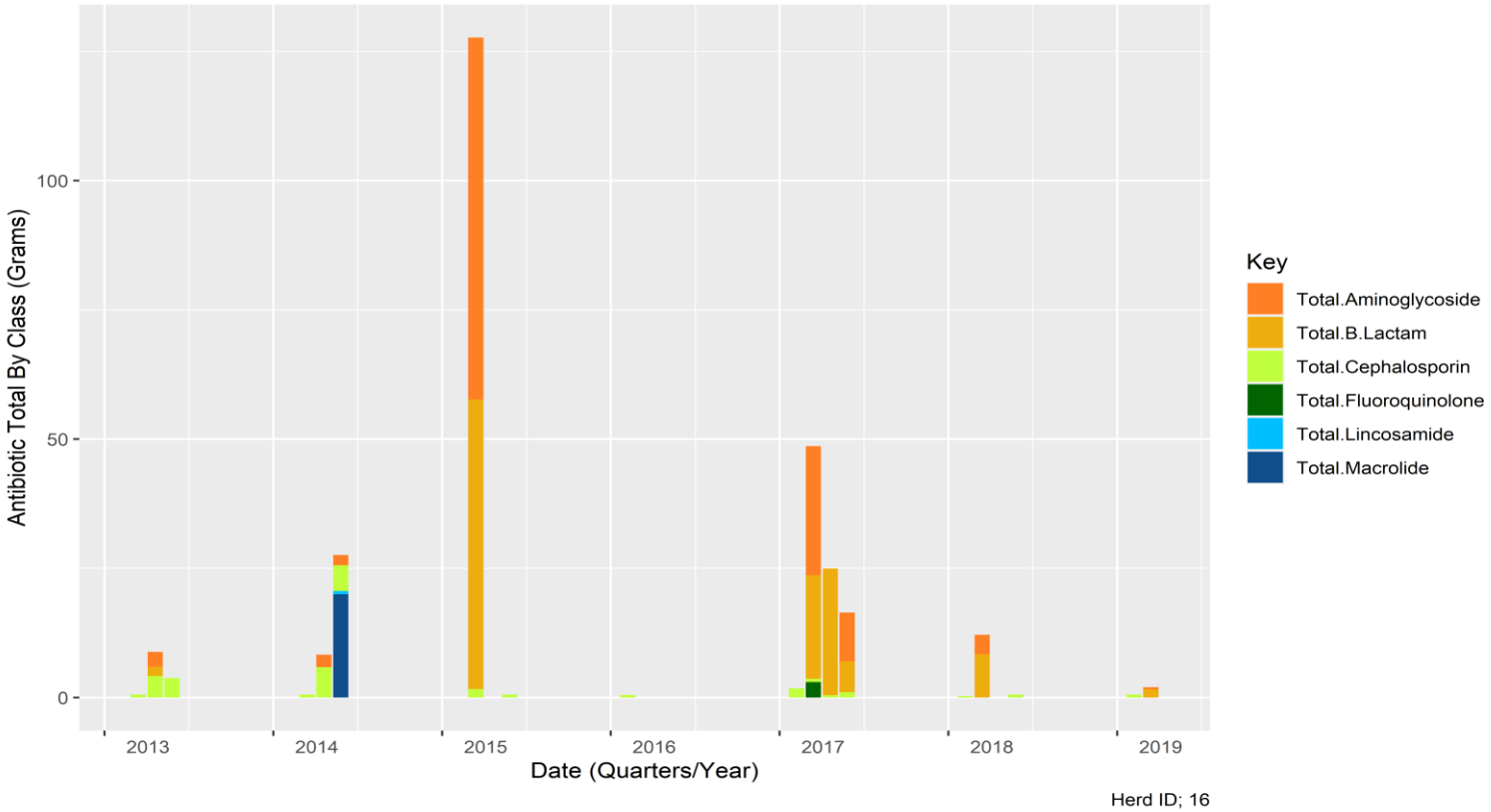

Figure A2.16. Graphical illustration of AMU for Farm 16 across the AMU sampling period between April 2013 and April 2019, presented in terms of total antimicrobial class used per quarter per year.

Table A2.1**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=88) from bulk tank samples (n=16) received August 2014 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						1	5	10	27	45
Amoxicillin/Clavulanic Acid	0		12	40	36	-	-	-			
Ampicillin	0		7	4	61	16	-	-			
Cefazolin	100				-	-	-	-	-	30	58
Cefovecin [a]	-		-	-	-	1	-	87			
Cefpodoxime [a]	-				1	-	2	85			
Ceftazidime [a]	-						-	-	88		
Cephalexin [a]	-			-	-	-	-	-	88		
Chloramphenicol	0					-	44	44	-	-	-
Doxycycline	0		79	-	-	-	5	4			
Enrofloxacin [a]	-	-	1	12	39	7	29				
Gentamicin [a]	-		-	-	1	14	17	56			
Imipenem	16				51	23	14	-			
Marbofloxacin [a]	-	-	-	-	7	40	41				
Orbifloxacin [a]	-				-	14	43	31			
Piperacillin/Tazobactam [a]	-							72	16	-	-
Pradofloxacin [a]	-		25	32	10	21					
Tetracycline	10.2						79	-	9		
Trimethoprim/Sulfamethoxazole [a]	-			88	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.2**.** Distribution of the MICs of *Enterococcus spp.* isolated (n= 86) from bulk tank samples (n=16) received August 2015 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						2	14	12	16	42
Amoxicillin/Clavulanic Acid	0		20	29	37	-	-	-			
Ampicillin	0		14	9	61	2	-	-			
Cefazolin	100				-	-	-	2	6	41	37
Cefovecin [a]	-		-	-	-	2	4	81			
Cefpodoxime [a]	-				-	2	5	79			
Ceftazidime [a]	-						-	-	86		
Cephalexin [a]	-			-	-	-	-	-	86		
Chloramphenicol	0					-	60	26	-	-	-
Doxycycline	1.2		63	-	-	2	2	19			
Enrofloxacin [a]	-	-	6	6	51	8	15				
Gentamicin [a]	-		-	-	1	25	27	33			
Imipenem	2.3				71	13	4	-			
Marbofloxacin [a]	-	-	-	4	7	52	23				
Orbifloxacin [a]	-				4	9	49	24			
Piperacillin/Tazobactam [a]	-							84	2	-	-
Pradofloxacin [a]	-		33	32	10	11					
Tetracycline	26.7						63	-	23		
Trimethoprim/Sulfamethoxazole [a]	-			86	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.3**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=87) from bulk tank samples (n=16) received August 2016 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						6	10	3	9	59
Amoxicillin/Clavulanic Acid	0		6	61	20	-	-	-			
Ampicillin	0		1	15	71	-	-	-			
Cefazolin	100				-	-	-	-	-	35	52
Cefovecin [a]	-		-	-	-	-	-	87			
Cefpodoxime [a]	-				-	-	3	84			
Ceftazidime [a]	-						-	-	87		
Cephalexin [a]	-			-	-	-	-	-	87		
Chloramphenicol	2.3					11	58	16	-	-	2
Doxycycline	1.2		72	-	-	1	1	13			
Enrofloxacin [a]	-	-	4	16	35	7	25				
Gentamicin [a]	-		-	1	5	10	6	28			
Imipenem	4.6				65	18	4	-			
Marbofloxacin [a]	-	-	-	3	2	50	32				
Orbifloxacin [a]	-				3	6	45	9			
Piperacillin/Tazobactam [a]	-							85	2	-	-
Pradofloxacin [a]	-		31	28	6	22					
Tetracycline	17.2						72	-	15		
Trimethoprim/Sulfamethoxazole [a]	-			77	7	-	3				

[a] Clinical breakpoints for resistance not defined

Table A2.4**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=90) from bulk tank samples (n=16) received August 2017 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						1	4	10	17	58
Amoxicillin/Clavulanic Acid	0		20	47	22	1	-	-			
Ampicillin	0		8	23	58	-	1	-			
Cefazolin	100				-	-	-	-	7	42	41
Cefovecin [a]	-		-	-	1	-	3	86			
Cefpodoxime [a]	-				1	1	9	79			
Ceftazidime [a]	-						1	-	89		
Cephalexin [a]	-			-	-	-	-	-	90		
Chloramphenicol	2.2					1	62	25	-	1	1
Doxycycline	8.9		57	-	1	1	6	25			
Enrofloxacin [a]	-	-	2	26	32	6	26				
Gentamicin [a]	-		-	-	1	10	20	59			
Imipenem	5.5				64	21	4	1			
Marbofloxacin [a]	-	-	-	2	21	39	28				
Orbifloxacin [a]	-				1	26	36	27			
Piperacillin/Tazobactam [a]	-							87	3	-	-
Pradofloxacin [a]	-		46	20	6	18					
Tetracycline	37.8						56	-	34		
Trimethoprim/Sulfamethoxazole [a]	-			90	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.5**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=89) from bulk tank samples (n=16) received August 2018 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						-	-	12	12	65
Amoxicillin/Clavulanic Acid	0		13	60	15	2	-	-			
Ampicillin	0		4	27	53	4	1	-			
Cefazolin	100				-	-	-	-	7	48	34
Cefovecin [a]	-		-	-	-	1	2	86			
Cefpodoxime [a]	-				3	1	4	81			
Ceftazidime [a]	-						-	-	89		
Cephalexin [a]	-			-	-	-	-	-	89		
Chloramphenicol	3.4					-	66	20	-	-	3
Doxycycline	4.5		62	-	-	1	10	16			
Enrofloxacin [a]	-	-	5	19	48	1	16				
Gentamicin [a]	-		-	-	-	7	24	58			
Imipenem	14.6				70	6	11	2			
Marbofloxacin [a]	-	-	-	6	17	49	17				
Orbifloxacin [a]	-				3	20	48	18			
Piperacillin/Tazobactam [a]	-							82	7	-	-
Pradofloxacin [a]	-		56	16	1	16					
Tetracycline	30.3						62	-	27		
Trimethoprim/Sulfamethoxazole [a]	-			89	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.6. Distribution of the MICs of *Enterococcus spp.* isolated (n=96) from bulk tank samples (n=16) received November 2018 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						-	1	13	15	67
Amoxicillin/Clavulanic Acid	0		11	58	26	-	-	1			
Ampicillin	1.1		3	12	74	6	-	1			
Cefazolin	100				-	-	-	2	4	48	42
Cefovecin [a]	-		-	-	1	-	-	95			
Cefpodoxime [a]	-				1	-	4	91			
Ceftazidime [a]	-						1	-	95		
Cephalexin [a]	-			-	-	-	-	-	96		
Chloramphenicol	4.2					-	51	41	-	-	4
Doxycycline	0		79	1	-	-	3	13			
Enrofloxacin [a]	-	-	-	20	50	5	21				
Gentamicin [a]	-		-	-	-	5	19	72			
Imipenem	20.8				69	7	20	-			
Marbofloxacin [a]	-	-	-	6	9	54	27				
Orbifloxacin [a]	-				-	20	52	24			
Piperacillin/Tazobactam [a]	-							86	9	1	-
Pradofloxacin [a]	-		40	34	-	22					
Tetracycline	16.7						80	-	16		
Trimethoprim/Sulfamethoxazole [a]	-			96	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.7**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=89) from bulk tank samples (n=16) received February 2019 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						5	-	4	5	75
Amoxicillin/Clavulanic Acid	0		2	45	41	1	-	-			
Ampicillin	0		1	3	81	3	1	-			
Cefazolin	100				-	-	-	-	-	56	33
Cefovecin [a]	-		-	-	-	-	-	89			
Cefpodoxime [a]	-				-	-	4	89			
Ceftazidime [a]	-						-	-	89		
Cephalexin [a]	-			-	-	-	-	-	89		
Chloramphenicol	4.5					-	13	72	-	-	4
Doxycycline	4.5		66	1	-	-	5	17			
Enrofloxacin [a]	-	-	-	15	57	4	13				
Gentamicin [a]	-		-	-	4	1	6	78			
Imipenem	4.5				76	9	2	2			
Marbofloxacin [a]	-	-	-	1	7	70	11				
Orbifloxacin [a]	-				1	18	59	11			
Piperacillin/Tazobactam [a]	-							81	6	2	-
Pradofloxacin [a]	-		50	27	2	10					
Tetracycline	27						65	-	24		
Trimethoprim/Sulfamethoxazole [a]	-			88	-	1	-				

[a] Clinical breakpoints for resistance not defined

Table A2.8. Distribution of the MICs of *Enterococcus spp.* isolated (n= 90) from bulk tank samples (n=16) received August 2019 via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	-						-	9	15	12	54
Amoxicillin/Clavulanic Acid	0		11	53	26	-	-	-			
Ampicillin	0		10	7	69	4	-	-			
Cefazolin	100				-	-	-	-	7	43	40
Cefovecin [a]	-		-	-	-	-	1	89			
Cefpodoxime [a]	-				-	1	4	85			
Ceftazidime [a]	-						-	-	90		
Cephalexin [a]	-			-	-	-	-	-	90		
Chloramphenicol	1.1					-	56	33	-	-	1
Doxycycline	0		76	-	-	-	3	11			
Enrofloxacin [a]	-	-	3	11	43	14	19				
Gentamicin [a]	-		-	-	1	18	13	58			
Imipenem	11.1				61	19	9	1			
Marbofloxacin [a]	-	-	-	3	1	52	36				
Orbifloxacin [a]	-				1	9	53	27			
Piperacillin/Tazobactam [a]	-							76	14	-	-
Pradofloxacin [a]	-		26	44	10	10					
Tetracycline	15.5						76	-	14		
Trimethoprim/Sulfamethoxazole [a]	-			90	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.9. Distribution of the MICs of *E. coli* isolated (n=90) from bulk tank samples (n=16) received August 2018 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	0						90	3	-	-	-
Amoxicillin/Clavulanic Acid	0		-	-	-	34	47	12			
Ampicillin	10.8		-	-	8	34	41	10			
Cefazolin	3.2				3	74	13	3	-	-	-
Cefovecin [a]	-		7	49	36	1	-	-			
Cefpodoxime [a]	0				92	1	-	-			
Ceftazidime [a]	0						92	1	-		
Cephalexin [a]	-			-	-	-	47	46	-		
Chloramphenicol	0					1	20	70	2	-	-
Doxycycline	0		-	1	42	45	4	1			
Enrofloxacin [a]	-	93	-	-	-	-	-				
Gentamicin [a]	0		-	35	55	3	-	-			
Imipenem	1.1				92	-	1	-			
Marbofloxacin [a]	-	91	1	1	-	-	-				
Orbifloxacin [a]	-				93	-	-	-			
Piperacillin/Tazobactam [a]	0							93	-	-	-
Pradofloxacin [a]	-		93	-	-	-					
Tetracycline	3.2						90	-	3		
Trimethoprim/Sulfamethoxazole [a]	0			93	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.10. Distribution of the MICs of *E. coli* isolated (n=92) from bulk tank samples (n=16) received November 2018 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	0						91	1	-	-	-
Amoxicillin/Clavulanic Acid	0		-	-	-	13	61	18			
Ampicillin	6.5		-	-	3	38	44	7			
Cefazolin	2.2				1	71	18	2	-	-	-
Cefovecin [a]	-		6	41	42	3	-	-			
Cefpodoxime [a]	0				91	1	-	-			
Ceftazidime [a]	0						92	-	-		
Cephalexin [a]	-			-	-	-	26	58	8		
Chloramphenicol	0					-	10	76	6	-	-
Doxycycline	4.3		-	2	27	54	4	5			
Enrofloxacin [a]	-	92	-	-	-	-	-				
Gentamicin [a]	0		-	46	43	3	-	-			
Imipenem	0				92	-	-	-			
Marbofloxacin [a]	-	90	1	1	-	-	-				
Orbifloxacin [a]	-				92	-	-	-			
Piperacillin/Tazobactam [a]	0							92	-	-	-
Pradofloxacin [a]	-		92	-	-	-					
Tetracycline	4.3						88	-	4		
Trimethoprim/Sulfamethoxazole [a]	4.3			88	-	-	4				

[a] Clinical breakpoints for resistance not defined

Table A2.11. Distribution of the MICs of *E. coli* isolated (n=94) from bulk tank samples (n=16) received February 2019 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	4.3						85	4	1	-	4
Amoxicillin/Clavulanic Acid	7.4		-	-	1	31	46	16			
Ampicillin	9.6		1	-	13	45	24	11			
Cefazolin	7.4				3	72	12	1	1	5	-
Cefovecin [a]	-		7	59	21	2	-	5			
Cefpodoxime [a]	5.3				89	-	-	5			
Ceftazidime [a]	4.3						89	1	4		
Cephalexin [a]	-			-	-	1	45	41	7		
Chloramphenicol	5.3					-	11	71	7	-	5
Doxycycline	7.4		-	3	38	41	3	9			
Enrofloxacin [a]	-	87	5	1	-	1	-				
Gentamicin [a]	4.3		1	56	26	7	-	4			
Imipenem	0				94	-	-	-			
Marbofloxacin [a]	-	86	2	2	4	-	-				
Orbifloxacin [a]	-				90	4	-	-			
Piperacillin/Tazobactam [a]	0							89	-	4	1
Pradofloxacin [a]	-		94	-	-	-					
Tetracycline	10.7						84	-	10		
Trimethoprim/Sulfamethoxazole [a]	3.2			91	-	-	3				

[a] Clinical breakpoints for resistance not defined

Table A2.12. Distribution of the MICs of *E. coli* isolated (n=87) from bulk tank samples (n=16) received August 2019 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	0						83	4	-	-	-
Amoxicillin/Clavulanic Acid	0		-	-	1	33	52	1			
Ampicillin	0		-	-	7	54	25	1			
Cefazolin	0				4	76	7	-	-	-	-
Cefovecin [a]	-		7	55	24	1	-	-			
Cefpodoxime [a]	0				87	-	-	-			
Ceftazidime [a]	0						87	-	-		
Cephalexin [a]	-			-	-	-	33	53	1		
Chloramphenicol	0					-	12	73	2	-	-
Doxycycline	4.6		-	3	31	49	-	4			
Enrofloxacin [a]	-	87	-	-	-	-	-				
Gentamicin [a]	0		-	37	45	5	-	-			
Imipenem	0				87	-	-	-			
Marbofloxacin [a]	-	87	-	-	-	-	-				
Orbifloxacin [a]	-				87	-	-	-			
Piperacillin/Tazobactam [a]	0							87	-	-	-
Pradofloxacin [a]	-		87	-	-	-					
Tetracycline	4.6						83	-	4		
Trimethoprim/Sulfamethoxazole [a]	0			87	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.13. Distribution of the MICs of *E. coli* isolated (n=82) from bulk tank samples (n=16) received November 2019 determined via the COMPGNF1 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Amikacin [a]	1.2						80	1	-	-	1
Amoxicillin/Clavulanic Acid	0		-	-	2	27	50	3			
Ampicillin	1.2		-	-	6	50	23	3			
Cefazolin	1.2				1	72	8	-	-	1	-
Cefovecin [a]	-		4	50	24	2	1	1			
Cefpodoxime [a]	1.2				81	-	-	1			
Ceftazidime [a]	1.2						81	-	1		
Cephalexin [a]	-			-	-	-	34	41	7		
Chloramphenicol	0					-	7	74	1	-	-
Doxycycline	0		-	2	17	62	1	-			
Enrofloxacin [a]	-	81	1	-	-	-	-				
Gentamicin [a]	1.2		-	42	34	5	-	1			
Imipenem	0				82	-	-	-			
Marbofloxacin [a]	-	80	1	1	-	-	-				
Orbifloxacin [a]	-				81	1	-	-			
Piperacillin/Tazobactam [a]								82	-	-	-
Pradofloxacin [a]	-		82	-	-	-					
Tetracycline	0						82	-	-		
Trimethoprim/Sulfamethoxazole [a]	0			82	-	-	-				

[a] Clinical breakpoints for resistance not defined

Table A2.14. Distribution of the MICs of *Enterococcus spp.* isolated (n=88) from bulk tank samples (n=16) received August 2014 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	4	4	80		
Cefquinome [a]	-				4	4	29	6			
Erythromycin	10.2	16	3	11	19	23	16				
Kanamycin/Cephalexin [a]	-						3	4	15	39	27
Oxacillin [a]	-				-	1	87				
Penicillin G	0	-	5	-	2	35	43	3			
Pirlimycin [a]	-				26	10	46				

[a] Clinical breakpoints for resistance not defined

Table A2.15**.** Distribution of the MICs of *Enterococcus spp.* isolated (n=86) from bulk tank samples (n=16) received August 2015 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	2	6	78		
Cefquinome [a]	-				7	5	26	48			
Erythromycin	1.2	10	3	12	35	23	3				
Kanamycin/Cephalexin [a]	-						1	4	11	54	16
Oxacillin [a]	-				-	1	85				
Penicillin G	0	-	3	5	4	48	25	1			
Pirlimycin [a]	-				21	11	54				

[a] Clinical breakpoints for resistance not defined

Table A2.16. Distribution of the MICs of *Enterococcus spp.* isolated (n=87) from bulk tank samples (n=16) received August 2016 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	-	6	81		
Cefquinome [a]	-				9	3	32	43			
Erythromycin	5.7	7	3	18	26	27	6				
Kanamycin/Cephalexin [a]	-						-	2	7	35	43
Oxacillin [a]	-				-	-	87				
Penicillin G	0	-	-	-	-	51	30	6			
Pirlimycin [a]	-				31	12	54				

[a] Clinical breakpoints for resistance not defined

Table A2.17. Distribution of the MICs of *Enterococcus spp.* isolated (n=88) from bulk tank samples (n=16) received August 2017 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	-	1	89		
Cefquinome [a]	-				1	6	24	59			
Erythromycin	16.7	10	-	8	24	29	19				
Kanamycin/Cephalexin [a]	-						1	7	7	40	35
Oxacillin [a]	-				-	1	89				
Penicillin G	1.1	-	-	1	5	45	36	3			
Pirlimycin [a]	-				27	9	54				

[a] Clinical breakpoints for resistance not defined

Table A2.18. Distribution of the MICs of *Enterococcus spp.* isolated (n=89) from bulk tank samples (n=16) received August 2018 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	-	-	89		
Cefquinome [a]	-				-	2	23	64			
Erythromycin	10.1	6	8	13	37	13	3				
Kanamycin/Cephalexin [a]	-						-	2	10	41	36
Oxacillin [a]	-				-	-	89				
Penicillin G	5.6	-	-	-	2	36	41	10			
Pirlimycin [a]	-				15	14	60				

[a] Clinical breakpoints for resistance not defined

Table A2.19. Distribution of the MICs of *Enterococcus spp.* isolated (n=96) from bulk tank samples (n=16) received November 2018 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					6	-	4	86		
Cefquinome [a]	-				1	5	43	47			
Erythromycin	5.2	8	6	26	31	19	6				
Kanamycin/Cephalexin [a]	-						-	5	12	41	38
Oxacillin [a]	-				-	1	95				
Penicillin G	1	-	-	-	7	63	21	5			
Pirlimycin [a]	-				36	7	53				

[a] Clinical breakpoints for resistance not defined

Table A2.20. Distribution of the MICs of *Enterococcus spp.* isolated (n=89) from bulk tank samples (n=16) received February 2019 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					-	-	-	89		
Cefquinome [a]	-				-	2	40	47			
Erythromycin	8.9	4	2	11	42	19	11				
Kanamycin/Cephalexin [a]	-						1	-	15	46	27
Oxacillin [a]	-				-	-	89				
Penicillin G	1.1	-	-	-	1	58	28	2			
Pirlimycin [a]	-				14	6	69				

[a] Clinical breakpoints for resistance not defined

Table A2.21. Distribution of the MICs of *Enterococcus spp.* isolated (n=90) from bulk tank samples (n=16) received August 2019 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	-					2	-	-	88		
Cefquinome [a]	-				2	1	28	59			
Erythromycin	4.5	17	3	11	22	23	14				
Kanamycin/Cephalexin [a]	-						4	2	10	47	27
Oxacillin [a]	-				-	-	90				
Penicillin G	0	2	-	-	6	40	35	7			
Pirlimycin [a]	-				25	11	54				

[a] Clinical breakpoints for resistance not defined

Table A2.22. Distribution of the MICs of *E. coli* isolated (n=93) from bulk tank samples (n=16) received August 2018 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	0					93	-	-	-		
Cefquinome [a]	-				93	-	-	-			
Erythromycin	-	-	-	-	-	-	93				
Kanamycin/Cephalexin [a]	-						88	2	-	-	88
Oxacillin [a]	-				-	-	93				
Penicillin G	-	-	-	-	-	-	-	93			
Pirlimycin [a]	-				-	-	93				

[a] Clinical breakpoints for resistance not defined

Table A2.23. Distribution of the MICs of *E. coli* isolated (n=92) from bulk tank samples (n=16) received November 2018 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	0					92	-	-	-		
Cefquinome [a]	-				92	-	-	-			
Erythromycin	-	-	-	-	-	-	92				
Kanamycin/Cephalexin [a]	-						87	5	-	-	-
Oxacillin [a]	-				92	-	-				
Penicillin G	-	-	-	-	-	-	-	92			
Pirlimycin [a]	-				-	-	92				

[a] Clinical breakpoints for resistance not defined

Table A2.24. Distribution of the MICs of *E. coli* isolated (n=94) from bulk tank samples (n=16) received February 2019 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	0					92	2	3	-		
Cefquinome [a]	-				89	-	2	3			
Erythromycin	-	-	-	-	-	-	94				
Kanamycin/Cephalexin [a]	-						89	4	1	-	-
Oxacillin [a]	-				-	-	94				
Penicillin G	-	-	-	-	-	-	-	94			
Pirlimycin [a]	-				-	-	94				

[a] Clinical breakpoints for resistance not defined

Table A2.25. Distribution of the MICs of *E. coli* isolated (n=87) from bulk tank samples (n=16) received August 2019 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	0					87	-	-	-		
Cefquinome [a]	-				87	-	-	-			
Erythromycin	-	-	-	-	-	1	86				
Kanamycin/Cephalexin [a]	-						86	1	-	-	-
Oxacillin [a]	-				-	-	87				
Penicillin G	-	-	-	-	-	-	-	87			
Pirlimycin [a]	-				-	-	87				

[a] Clinical breakpoints for resistance not defined

Table A2.26. Distribution of the MICs of *E. coli* isolated (n=82) from bulk tank samples (n=16) received November 2019 determined via Mastitis-3 microdilution plates alongside resistance percentage of each antimicrobial. Shading indicates that corresponding MIC values were not determined.

		Number of isolates corresponding to MIC values (µg/ml)									
Antimicrobial	**% of isolates deemed resistant**	**≤0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>32**
Cefoperazone [a]	0					81	-	-	1		
Cefquinome [a]	-				80	1	-	1			
Erythromycin	-	-	-	-	-	-	82				
Kanamycin/Cephalexin [a]	-						80	1	-	-	1
Oxacillin [a]	-				-	-	82				
Penicillin G	-	-	-	-	-	-	-	82			
Pirlimycin [a]	-				-	-	82				

[a] Clinical breakpoints for resistance not defined

Appendix – Chapter 3

Table A3.1. An overview of the final dataset of questionnaire responses used for analysis for study group 1 farms, including lists of possible responses and codes assigned for analysis purposes.

Question No.	Question	Possible Responses	Response Code
1(a)	Average annual herd size	Numerical response	N/A
1(b)	Average milk sales (litres) in past year		
1(c)	Average milk sales (litres/cow/year in past year)		
1(d)	Stocking rate		
2	Milking frequency	Twice a day	1
		Three times a day	2
		Robotic milking	3
3	No. of milking parlour units	1-12	1
		13-24	2
		25-36	3
		37-48	4
		49-60	5
4	Milking parlour age	<1 year	1
		1-6 years	2
		6-10 years	3
		>10 years	4
5(a)	Are clusters disinfected between use?	Yes	50
		No	60
5(b)	Type of cluster disinfection	Not applicable	1
		Manual (some)	2
		Manual (all)	3
		Automatic	4
5(c)	Is the milking plant given a hot wash every milking?	Yes	50
		No	60
6	Are cows always stripped before being milked?	Yes	50
		No	60
7	What type of teat preparation occurs at milking?	Pre dipping (with wipe)	1
		Brushed	2
		Dry wiped	3
		Wet wiped	4

		Unknown	5
		washed	6
8	Ventilation score of housing	Numerical response	N/A
9	Cubicle bedding type	Sawdust	1
		Sand	2
		RMS (recycled manure solids)	3
10	Cubicle type	Mat	1
		Deep bedding	2
		Both	3
11	Frequency of bedding	2-3 times per day	1
		Daily	2
		2-6 times per week	3
		Weekly or less frequently	4
12	Are bedding conditioners used?	Yes	50
		No	60
13	Are conditioners stored or left open?	Yes	50
		No	60
14(a)	Farm location within UK (region)	South West	SW
		South East	SE
		North West	NW
		North East	NE
		Mid-West	MW
		Mid-East	ME

Table A3.2. An overview of the final dataset of questionnaire responses used for analysis for study group 2 farms, including lists of possible responses and codes assigned for analysis purposes.

Question No.	Question	Possible Responses	Response Code
1a (I)	No. of adult dairy cows in herd	Numerical response	N/A
1a (II)	Annual average of dairy cows		
1b (I)	No. of dairy cows in milk		
1b (II)	Annual average of dairy cows in milk		
1c (I)	No. of dry cows		
1c (II)	Annual average of dry cows		
1d (I)	No. of pregnant heifers		
1d (II)	Annual average of pregnant heifers		
1e (I)	No. of other dairy youngstock		
1e (II)	Annual average of dairy youngstock		
1f (I)	No. of beef cattle (if applicable)		
1f (II)	Annual average of beef cattle (if applicable)		
1g (I)	No. of sheep (if applicable)		
1g (II)	Annual average of sheep (if applicable)		
1h (I)	No. of non-ruminant animals (if applicable)		
1h (II)	Annual average of non-ruminants (if applicable)		
2	Pig or Poultry units nearby?	Yes	50
		No	60
3	Are animals bought into/sold off farm?	Yes	50
		No	60
4	Has the adult dairy herd changed significantly in last 12 months?	Yes	50
		No	60
		An increase	2
		A decrease	3
5	Method of dairy cattle reproduction	Bull brought in to farm from elsewhere	1

		Bull reared on farm	2
		Artificial insemination always	3
6	Annual milk yield (litres/cow/year)	Numerical response	N/A
7	Typical monthly SCC levels	< 50,000	1
		50,000 – 100,000	2
		100,000 – 150,000	3
		150,000 – 200,000	4
		200,000 – 300,000	5
8	Typical monthly Bactoscan levels	< 20,000	1
		20,000 – 30,000	2
		30,000 – 40,000	3
		40,000 – 50,000	4
		50,000 – 100,000	5
		100,000 – 150,000	6
9	Does milking parlour have backflush wash?	Yes	50
		No	60
10	How many times are milk liners changed per year?	Numeric response	N/A
11	Are teats dry wiped before milking?	Yes	50
		No	60
12	Are teats wet wiped before milking?	Yes	50
		No	60
13	Are teats disinfected before milking?	Yes	50
		No	60
14	Are teats disinfected after milking?	Yes	50
		No	60
15	Are disposable gloves worn during milking?	Yes	50
		No	60
16	Are gloves washed during milking?	Yes	50
		No	60
17	When are gloves washed (if applicable)	After each row	1
		Throughout milking	2
		Dependent on cleanliness	3
		Not applicable	4
18	Is selective dry cow therapy employed?	Yes	50
		No	60
19	Antibiotic dry cow tubes always used	Yes	50
		No	60
20	Natural drying off occurs in some/all cows	Yes	50
		No	60
21		Yes	50

	Mastitis treatment – antibiotic tubes always used	No	60
22	Mastitis treatment – injectable antibiotics always used	Yes	50
		No	60
23	Mastitis treatment – anti inflammatory always used	Yes	50
		No	60
24	Mastitis treatment – homeopathic remedies used	Yes	50
		No	60
25	Are wormers used in dairy cattle?	Yes	50
		No	60
26	If yes, what animals are wormed?	Youngstock	1
		Cows	2
		Youngstock and cows	3
		None used	4
27	How are medicine records kept?	Paper	1
		Electronically	2
		Paper & electronically	3
28	How are medicines purchased?	From vet	1
		Online	2
		Both	3
29	When are cows turned out to graze?	March	1
		April	2
		May	3
		Grazing most of the year	4
30	When do cows return to housing for the winter period?	October	1
		November	2
		December	3
		Grazing most of the year	4
31	Type of cubicle matting used in housing	Rubber mat	1
		Mattress	2
		Water bed	3
		Not applicable (concrete flooring)	4
32	Type of cubicle bedding used	Straw	1
		Sawdust	2
		Sand	3
		Not applicable	4
33	Antibacterial materials used in bedding	Yes	50
		No	60
34	Product used	None used	1
		Antibacter	2
		Hydrated lime	3

35	Frequency of antibacterial material application	None used	1
		Twice daily	2
		Once daily	3
		Every other day or more	4
36	How often are cubicles scraped clean of manure?	Not applicable	1
		Three times daily	2
		Twice daily	3
		Once daily	4
37	How often is cubicle bedding reapplied?	Not applicable	1
		Twice daily	2
		Once daily	3
		Every other day	4
		More than every other day	5
38	How often are passageways scraped of manure?	Three or more times daily	1
		Twice daily	2
		Once daily	3
		Every other day	4
39	Within the last 12 months, have any housing management changed significantly?	Yes	50
		No	60
40	If changes have been made, what areas have changed?	Change of bedding routine	1
		Increased scraping frequency	2
		No changes	3
41(a)	Do you consider pneumonia to be a problem on farm?	Yes	50
		No	60
41(b)	How is this treated?	Not a problem	100
		Antibiotics	1
		Other	2
43(a)	Is diarrhoea/scour considered a problem?	Yes	50
		No	60
43(b)	How is this treated?	Not a problem	100
		Oral rehydration therapy	1
		Antibiotics	2
		Both	3
44	How is waste milk dealt with?	Feeding calves	1
		Dumped in slurry pit	2
		Both	3
		Other	4
45(a)	Where is water for use in the parlour sourced?	Mains supply	1
		Borehole	2
45(b)	What chemicals are used for washing of the	Sodium hydroxide/sodium hypochlorite	1

	bulk tank? (By active ingredients)	Sodium hydroxide/sodium carbonate	2
		Phosphoric/sulphonic acid	3
		Sodium hydroxide/phosphoric acid	4
45(c)	What chemicals are used for washing of the milking parlour (By active ingredients)	Sodium hydroxide/sodium hypochlorite	1
		Sodium hydroxide/sodium carbonate	2
		Phosphoric/sulphonic acid	3
		Sodium hydroxide/phosphoric acid	4
45(d)	What products (by active ingredient) are used for foot bathing?	Formaldehyde	1
		Zinc & copper	2
		No foot bathing	3
46(a)	Are above ground slurry stores present on farm?	Yes	50
		No	60
46(b)	If yes, when is this emptied?	Dependent on ground conditions	1
		Twice a year	2
		Not applicable	3
46(c)	What land is spread with slurry?	All land	1
		Grazing land only	2
		Silage land only	3
		Other	4
47(a)	How is solid waste bedding handled?	Stored at dung heap	1
		Spread on land straight away	2
47(b)	What land is spread with solid waste bedding?	All land	1
		Grazing land only	2
		Silage land only	3
		other	4