# Prevalence of Antibiotic Resistance in Gram-Positive Bacteria from Raw Milk, and Antibiotic Resistance Gene Analysis in a Mastitis-Associated *Streptococcus uberis* Isolate from New Zealand

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# Abstract

Antimicrobial resistance is a growing global issue, with resistance to antibiotics increasing at a concerning rate. Resistance is an issue in both human and animal infection which has considerable implications for both human health and the economy. On dairy farms in New Zealand, antibiotic resistant bacteria can be found in the environment or as part of a cow's natural microflora. If cattle become ill with mastitis, a mammary infection caused by a range of Gram-positive and Gram-negative bacteria, treatment with antibiotics can become complex if resistant bacteria are present. Therefore, antibiotic resistant bacteria surveillance in NZ dairy cattle is an important aspect of combatting this issue.

Antibiotic susceptibility testing on raw milk from NZ dairy farms using the Kirby-Bauer disc diffusion method was undertaken to measure the frequency of resistant bacteria. The extraction of bacterial DNA was performed with the intention of investigating the genome for genes that match its phenotypic resistance profile. Moreover, genomic analysis of the bovine mastitis isolate *Streptococcus uberis* was performed, using CARD and BLAST, to identify any relevant antibiotic resistance genes.

Raw milk testing identified some putative *Staphylococcus* and *Streptococcus/Enterococcus* which demonstrated antibiotic resistance. Both the *Staphylococcus* and *Streptococcus*/*Enterococcus* isolates displayed 100% resistance to penicillin G. *Staphylococcus* general has a high level of resistance to β-lactam antibiotics whereas *Streptococcus*/*Enterococcus* were highly susceptible to the other β-lactams. *Streptococcus*/*Enterococcus* had moderate levels of resistance to novobiocin. The methicillin-resistant *Staphylococcus aureus* (MRSA) did not diverge from the expected resistance profile. The quality control *S. aureus* had some atypical results, which may suggest phenotypic changes while in storage.

The CARD analysis for *S. uberis* found three antibiotic resistance genes, *lnu*(C) and *patA*/*patB*. BLAST analysis and a literature search showed *lnu*(C) is likely to be present in the *S. uberis* isolate but *patA*/*patB* is less likely to be present. In order to prove their presence and activity in the bacterium, further susceptibility testing and genome analysis is required.

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# Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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# Abbreviations

| | |
|---|---|
| % | Percentage |
| β | Beta |
| °C | Degrees Celcius |
| g | Gram |
| *g* | Gravity |
| kPa | Kilopascal |
| mL | Millilitre |
| mm | Millimetre |
| rpm | Revolutions per minute |
| V | Volts |
| µg | Microgram |
| µL | Microlitre |
| ABC | ATP-binding cassette superfamily |
| AMR | Antimicrobial resistance |
| ARB | Antibiotic resistant bacteria |
| ARG | Antibiotic resistance gene |
| AUT | Auckland University of Technology |
| bp | Base pairs |
| BPA | Baird Parker agar |
| BLAST | Basic local alignment search tool |
| CA | Clavulanic acid |
| CARD | Comprehensive antibiotic resistance database |
| CBA | Columbia agar with 5% sheep blood |
| CDS | Coding sequence |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Standards Institute |
| DNA | Deoxyribonucleic acid |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| GC | Guanine-cytosine |
| E | E-value |
| ID | Percentage identity |
| IS | Insertion sequence |
| MHA | Mueller-Hinton agar |
| MIC | Minimum inhibitory concentration |
| MPI | Ministry for Primary Industries |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| NA | Nutrient agar |
| NB | Nutrient broth |
| NZ | New Zealand |
| NZVA | New Zealand Veterinary Association |
| PBP | Penicillin-binding proteins |
| QC | Query cover |
| RAST | Rapid annotation using subsystem technology |
| RGI | Resistance gene identifier |
| RQ | Research question |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SNP | Single nucleotide polymorphism |
| TBE | Tris-Borate ethylenediaminetetraacetic acid |
| WHO | World Health Organization |

# 1.0 Introduction

## 1.1 General Introduction

Antibiotic resistance is widely recognised as an urgent global public health emergency (Stower, 2020). Though antibiotic resistance has been present almost as long as antibiotics themselves, it has been accelerated in part by inappropriate antibiotic use in both medical and agricultural fields (Hwang & Gums, 2016; Morehead & Scarbrough, 2018). Antibiotic resistance is a natural process occurring in bacteria due to mutation and horizontal gene transfer, resulting in the gain of resistance genes (von Wintersdorff et al., 2016). The proliferation of antibiotic resistant bacteria (ARB) has severe consequences for the treatment of human infection, and in turn animal and environmental health (Samreen et al., 2021). The presence of ARB in food-producing animals is problematic due to the potential for transmitting to humans via food products, human-animal contact, and shared natural resources (Tang et al., 2017). As such, exploring the dissemination of resistance genes and the creation of ARB is crucial to combating this crisis.

The use of antibiotics in livestock for disease treatment, growth promotion and prophylaxis is a well-known source of ARB and antibiotic residuals in the global environment (Samreen et al., 2021). Sub-therapeutic antibiotic doses in production animals creates unnatural selective pressure, which leads to the persistence of resistant bacteria in soils, wastewater, and food supply (Noyes et al., 2016; Ventola, 2015; von Wintersdorff et al., 2016). In dairy farming environments, bovine mastitis is the most prevalent infection in cows therefore the predominant cause of antibiotic usage (Bates et al., 2020). Bovine mastitis is the inflammation of the cow's udder tissue which is typically caused by bacterial infection of the teat and the subsequent release of leukocytes into the area (Shin et al., 2021). This infection is usually treated with beta-lactam antibiotics including penicillins such as cloxacillin and amoxicillin-based antibiotics, or various cephalosporins (Ministry for Primary Industries [MPI], 2019).

Common bovine mastitis pathogens on New Zealand dairy farms include *Streptococcus uberis, Streptococcus dysgalactiae, Staphylococcus aureus, Escherichia coli,* enterococci, and coagulase negative staphylococci (McDougall et al., 2014; Petrovski et al., 2011). Varying levels of penicillin resistance in mastitis-causing *S. aureus* in NZ have been reported by commercial laboratories, though numbers have been steadily declining in the last few decades (McDougall et al., 2014). Mastitis-causing *Streptococcus* spp. has traditionally been susceptible to β-lactams including penicillins (Haenni et al., 2018). However, rising minimum inhibitory concentration (MIC) doses of β-lactams for *S. uberis* have been recorded by veterinary laboratories across NZ (McDougall et al., 2020).

The New Zealand Antimicrobial Resistance Action Plan was released in 2017 in response to a commitment by the United Nations General Assembly to combat this growing crisis (Ministry of Health and MPI 2017). This plan emphasises the need to support research in this area by nominating it a priority action. Antimicrobial resistance (AMR) in human pathogens is monitored by the Institute for Environmental Science and Research Limited (n.d.), whereas MPI, along with the New Zealand Veterinary Association (NZVA) and other stakeholders, oversees antimicrobial use and AMR in animal agriculture (Ministry of Health and MPI, 2017).

Antibiotic resistant bacteria are a global threat to disease control in both animals and humans (Bryan & Hea, 2017). Understanding the prevalence and mechanisms behind antibiotic resistance in bovine mastitis pathogens is only part of the approach to combatting this urgent issue. The objective of this research is to gain insight into the presence of Gram-positive ARB in raw milk on NZ dairy farms, as well as investigating what antibiotic resistance genes (ARGs) may be present in mastitis-causing Gram-positive bacteria.

## 1.2 Research Questions and Aims

Research question (RQ) 1: What antibiotic-resistant (AMR) Gram-positive bacteria are present in raw milk samples from several North Island, New Zealand dairy farms?

> Aim 1.1: To determine if there are any Gram-positive antibiotic resistant bacteria present in the raw milk samples.
>
> > Objective 1.1.1: Antibiotic disc diffusion assays will be performed using select antibiotics on agar plates containing isolates that have been acquired from raw milk samples. Zone of inhibition data will be collected to assess antibiotic resistance and susceptibility.

RQ2: What ARGs are present in the genomes of mastitis-causing *S. uberis* from swab samples collected from NZ dairy cattle?

> Aim 2.1: To identify any known resistance genes in the *S. uberis* genomes using CARD and other bioinformatics techniques.
>
> > Objective 2.1: Use CARD to identify any potential ARGs present, then use BLASTn alongside reference coding sequences (CDS) to provide evidence of the presence of these genes within the *S. uberis* genomes.

# 2.0 Literature Review

## 2.1 Antibiotic Use on NZ Dairy Farms

Antimicrobial usage on NZ farms is considered low compared to other countries for which this data is available, and has stabilised over time due to heightened focus on antimicrobial stewardship within the veterinary profession (Hillerton et al., 2021). This is, in part, owed to the fact that using antimicrobials for the goal of growth promotion is not permitted under NZ legislation (Hillerton et al., 2017). Additionally, pasture-based agricultural systems where cows are kept in open air environments is likely to contribute to lower antimicrobial use as this decreases the spread of key pathogens (Bryan & Hea, 2017).

Dairy farms in NZ are home to several key pathogens. *Campylobacter jejuni, Listeria monocytogenes, Salmonella,* and *E. coli* O157 amongst others have been previously detected in NZ raw milk tanks (Marshall et al., 2016). However, the majority of antibiotics used on dairy farms are to treat mastitis infections (Burgess & French, 2017). The most frequently occurring bovine mastitis pathogens are *S. uberis, S. dysgalactiae, S. aureus, E. coli,* the enterococci, and coagulase-negative staphylococci (McDougall et al., 2014; Petrovski et al., 2011). For both streptococcal and staphylococcal mastitis, penicillins are the recommend antibiotic therapy; second line treatments for staphylococci include 1st generation cephalosporins and for streptococcal infections, a mixture of macrolide or tetracycline antibiotics may be used (NZVA, 2018). For Gram-negative infections, later generation cephalosporins or fluroquinolones are used, but given the frequent inability to rid cattle of some Gram-negative infections, culling may be necessary (NZVA, 2018).

## 2.2 Mastitis

Bovine mastitis is the inflammation of the cow's mammary glands, usually caused by infection by bacteria (Sharun et al., 2021). It results in reduced milk production due to tissue damage in the udder and poor milk quality, which has significant financial implications for farmers and other stakeholders (Brand et al., 2021). Mastitis can be classified as contagious, or environmental; and clinical, or sub-clinical (Hoekstra et al., 2020; Sharun et al., 2021). Contagious mastitis is the spread of contagious bacteria from infected to healthy cow, whereas environmental mastitis is the spread of pathogenic bacteria to healthy cows from environmental reservoirs such as bedding or soil (Sharun et al., 2021). Mastitis is clinical when the udder or the milk are noticeably abnormal; subclinical mastitis is infection without any observed abnormality (Hossain et al., 2015). Contagious mastitis-causing pathogens include *S. aureus, S. dysgalactiae* and *S. agalactiae,* whereas environmental mastitis infections are usually caused by *S. uberis* or Enterobacteriaceae (Cheng & Han, 2020; Sharun et al., 2021).

There are several different treatment options for bovine mastitis. The first method is antibiotic therapy, including dry cow therapy where cows are injected with antibiotic then the teats are sealed to prevent further infection. Other treatment options involve either vaccinations against the common pathogens, to culling in cases of chronic mastitis and severe infection with poor prognosis (Cheng & Han, 2020).

## 2.3 Antibiotics and AMR

Modern medicine has been transformed by the discovery and implementation of antibiotics, but the emergence of antibiotic resistance in conjunction with a lack of new antibiotics has created a worldwide health emergency (Alcock et al., 2020). Deaths due to infections by multidrug-resistant microorganisms are estimated to be approximately 700,000 deaths per annum (Ministry of Health and MPI, 2017) . Antibiotic resistance arises when bacteria no longer respond as expected to antibiotic therapy, usually because of mutation in the genome of the bacteria or the acquisition of AMR genes due to horizontal gene transfer (Ventola, 2015). Misuse of antibiotics contributes to resistance via selective pressure, whereby resistant bacteria are left to proliferate as those who are sensitive to antibiotic therapy are no longer able to compete with them (Ventola, 2015). Inappropriate antibiotic use is an issue in both human and agricultural medicine and as such, more responsible antibiotic stewardship in both arenas is required to combat this issue (Hwang & Gums, 2016).

### 2.3.1 Antibiotic Resistance and AMR in NZ

In relation to other countries, New Zealand has a low rate of AMR in spite of its higher than average antimicrobial usage (Royal Society Te Apārangi, 2017). However, sharp increases in methicillin-resistant *S. aureus* (MRSA) and extended-spectrum beta-lactamase (β-lactamase) producing *Enterobacteriaceae* infections, as well as the appearance of carbapenemase-producing *Enterobacterales* in New Zealand in clinical settings increases the need for vigilance around this topic (Heffernan et al., 2016; Institute of Environmental Science and Research Ltd, 2020; Moor et al., 2008). Resistance to antibiotics from the classes penicillins, fluoroquinolones and later generation cephalosporins, is common in both nosocomial and community settings (Williamson & Heffernan, 2014).

In animal agriculture, MPI and the NZVA monitor the sale and use of antimicrobials (Ministry of Health and MPI, 2017). However, surveillance on AMR in animals specifically is not carried out in NZ (Hillerton et al., 2017). Approximately 30% of *S. aureus* isolates found on NZ dairy farms are penicillin-resistant, however, this varies considerably from herd to herd (NZVA, 2018).

It is known that AMR bacteria can be spread from animals via the food chain, effluent in the environment and through direct contact between animals and humans (Woolhouse et al.,

2015). Unfortunately, the impact these factors have on humans is not well understood or quantified (Woolhouse et al., 2015). The lack of NZ specific data makes it difficult to create strong links between local antibiotic use and AMR in dairy cows, and AMR in humans; however, there is evidence that bacteria in the gut microflora of cows can develop AMR which could have implications for humans and the wider environment (Burgess & French, 2017).

### 2.3.2 Origins of AMR

There are two processes that result in acquired AMR in bacteria: by genetic mutation or horizonal gene transfer for relevant genes (Toombs-Ruane et al., 2017). Natural resistance can also occur, either intrinsically to the bacterium or induced in the bacterium after antibiotic exposure (Reygaert, 2018). Typically, resistance mechanisms are categorised by inhibiting the uptake of medicine, modifying the antibiotic target molecule, inactivating the medicine entirely, and the use of bacterial efflux pumps (Reygaert, 2018).

Genetic mutation in the chromosomal DNA can occur spontaneously, or in response to an external mutagenic agent (Coculescu, 2009). Mutations that encourage AMR typically occur in genes that encode drug targets, drug transporters and their regulators, as well as enzymatic modification of antibiotics (Reygaert, 2018). Mutations can be due to substitution, deletion, addition, inversion or duplication; if the mutation results in the production of a protein that confers resistance to antibiotics, the bacterium will survive antibiotic therapy and go on to proliferate with the mutation in effect (Coculescu, 2009). Environmental stressors, such as UV radiation, can often trigger genetic mutations in bacteria (Reygaert, 2018).

Horizontal gene transfer is the sharing of genetic material by way of conjugation, transformation, and transduction between unrelated organisms (Soucy et al., 2015). Resistance genes are transferred to other bacteria via genetic mobile elements or plasmids, with plasmid-associated transmission being the usual means of acquisition (Reygaert, 2018; Toombs-Ruane et al., 2017). Other mobile elements capable of transferring AMR genes include integrons and insertion sequences/transposons.

Transposons are genetic sequences that can alter their position within the DNA, therefore can be transferred between plasmids and between chromosomal DNA and plasmids (Babakhani & Oloomi, 2018). Like with mutation, the movement of transposons within the genome contribute to genetic variability which in turn can result in the emergence or development of bacterial resistance (Coculescu, 2009). For example, vancomycin-resistant enterococci are the result of transposon movement (Babakhani & Oloomi, 2018). As transposons containing AMR genes can contribute to the intra- and interspecies spread of resistance, understanding their

role in antibiotic resistance is part of the key to reducing the prevalence of AMR strains (Toombs-Ruane et al., 2017).

## 2.4 Overview of Antibiotics

### 2.4.1 Penicillin G

Penicillin G, also known as benzylpenicillin, is the first commercial therapeutic antibiotic known (MPI, 2019). It is a narrow-spectrum, bactericidal natural penicillin that is predominantly used to treat Gram-positive bacteria such as streptococci and non-penicillinase producing staphylococci, but can also be used against select Gram-negative cocci such as *Neisseria* (Yip & Gerriets, 2021). Penicillins are characterised by their β-lactam ring system, which is responsible for inactivating the transpeptidase reaction of the membrane-bound penicillin-binding proteins (PBP) between the terminal glycine residue and D-alanine which is necessary for the formation of bacterial cell-wall peptidoglycan (Soares et al., 2012). Pencillin G is used as a first line Gram-positive mastitis infection treatment on NZ dairy farms (NZVA, 2018). Penicillin resistance can occur due to the production of β-lactamases in bacteria, an enzyme that hydrolyses the peptide bond in the penicillin's β-lactam ring causing it to become ineffective (Majiduddin et al., 2002). Additionally, conformation changes in the PBP due to mutations in the genes can confer resistance (McGee et al., 2015). The discovery of β-lactamase-producing bacteria, such as *S. aureus* and *Enterobacteriaceae*, forced the development of antibiotics resistant to β-lactamases (Brook, 2009).

### 2.4.2 Ampicillin

Ampicillin is a broad-spectrum, semi-synthetic aminopenicillin that is used to treat both Gram-positive and negative bacterial infections in both humans and animals (World Health Organization [WHO], 2018). As a beta-lactam (β-lactam) antibiotic, ampicillin works similarly to penicillin by inhibiting cell wall synthesis due to the binding of the antibiotic to the PBP which eventually causes the cell to lyse and die (Suleyman & Zervos, 2016). The World Health Organization has classified ampicillin as a critically important antimicrobial for human medicine, therefore its use in livestock must be handled carefully as not to increase resistance to it (Food and Agriculture Organization of the United Nations & WHO, 2017). Ampicillin is used in conjunction with cloxacillin in NZ dairy cattle to treat intramammary infection in both dry and lactating cows (MPI, 2019). Like in penicillin, ampicillin resistance occurs due to the production of β-lactamases in bacteria, such as TEM-1 β-lactamase and ROB-1 β-lactamase, or confirmational changes in their PBP (Kim et al., 2007). Ampicillin can be used in conjunction with a β-lactamase inhibitor to extend its activity against bacteria that produce β-lactamases (MPI, 2019).

### 2.4.3 Amoxicillin + Clavulanic acid

Amoxicillin is an extended-spectrum, semi-synthetic aminopenicillin which was formulated to have greater effect against Gram-negative bacteria, as well as retain stability *in vivo* (MPI, 2019). As a part of the penicillin family, amoxicillin is susceptible to β-lactamases so can be combined with clavulanic acid which is a β–lactamase inhibiting compound. Amoxicillin + clavulanic acid, sometimes referred to as Augmentin, is also considered a critically important antimicrobial for human medicine by WHO (WHO, 2018). Clavulanic acid works due to enzyme inhibition, by binding the β-lactamase at its active site which prevents it from hydrolysing the β-lactam ring in the amoxicillin (Roy et al., 2018). Both amoxicillin and amoxicillin + clavulanic acid are used in NZ dairy cattle, though only amoxicillin by itself is used as a first line treatment for streptococcal mastitis as well as other infections (NZVA, 2018). Resistance to amoxicillin + clavulanic acid is usually due to the hyper-production of β-lactamases by bacteria, which quickly overwhelms the ability of clavulanic acid to inactivate the enzymes and defend the action of amoxicillin (MPI, 2019).

### 2.4.4 Cefuroxime

Cefuroxime is a broad-spectrum, second-generation cephalosporin that is effective against most Gram-positive cocci and somewhat effective against Gram-negative bacilli (Fernandes et al., 2013). Cephalosporins are part of the β–lactam class of antibiotics, but have a 6-sided dihydrothiazine β–lactam ring rather than penicillins' 5-sided thiazolidine ring as well as variations in side chain substituents; this confers a slightly elevated resistance to β–lactamases so can be used against penicillin-resistant bacteria (Chaudhry et al., 2019; El-Shaboury et al., 2007). Though structurally different, the mechanism of antibiotic action is the same as it is for the penicillins (Fernandes et al., 2013). Second-generation cephalosporins are considered 'orange light' antimicrobials by the NZ veterinary association, and should only be given as specific indications or as a second line therapy (NZVA, 2018). For streptococcal mastitis in NZ dairy cattle, 1$^{st}$ generation cephalosporins are recommended when penicillin has failed to cure the infection (NZVA, 2018). Resistance to cephalosporins is typically as it is with the penicillins, by β–lactamase hydrolysis or genetic changes in PBP. However, there is evidence that cefuroxime resistance can also occur due to the presence of efflux pumps (Källman et al., 2003).

### 2.4.5 Neomycin

Neomycin is a broad-spectrum, bactericidal aminoglycoside typically used to treat Gram-negative bacterial infections, though has some activity against Gram-positive organisms, especially staphylococci (Sasseville, 2010). Neomycin works through interfering with the 16S

rRNA by binding to the aminoacyl-tRNA site, which leads to codon misreading, mRNA mistranslation and eventually inhibition of bacterial protein synthesis (Fourmy et al., 1998; Mehta & Champney, 2003). Neomycin is also considered a critically important human antimicrobial by WHO (WHO, 2018). In NZ dairy cattle, neomycin is considered an 'orange light' antibiotic and is indicated for second line therapy for staphylococcal/coagulase-negative staphylococcal mastitis when penicillins have not worked (NZVA, 2018). Resistance to neomycin is typically conferred by aminoglycoside-modifying enzymes encoded the neomycin-resistance amino 3'-glycosyl phosphotransferase (*neo*) gene which modify and inactivate the antibiotic (Zhang et al., 2009).

### 2.4.6 Novobiocin

Novobiocin is a bactericidal, broad-spectrum aminocoumarin that is more often used in Gram-positive bacterial infections (Constable et al., 2017). Though effective against Gram-negative bacteria, due to the lipopolysaccharide-containing outer membrane acting as a barrier, novobiocin cannot always permeate the cell (May et al., 2017). Novobiocin's mechanism of action involves binding to the N-terminal section of the GyrB subunit of the bacterial DNA gyrase, blocking the ATPase reaction that initiates DNA supercoiling (Maxwell, 1993). Novobiocin, as well as aminocoumarins in general, are not currently approved for use in human medicine for systemic use (WHO, 2018). Though novobiocin was at one point used for mastitis treatment in NZ dairy cattle, it is not registered for veterinary use at this time (MPI, 2020; Salmon et al., 1998). Aside from the aforementioned resistance in Gram-negative bacteria, resistance to novobiocin is conferred by point mutations in the targeted subunit protein GyrB, which affects the binding ability of novobiocin (Vickers et al., 2007).

### 2.4.5 Vancomycin

Vancomycin is a narrow-spectrum, glycopeptide antibiotic typically used as a last-resort medication for MRSA and other serious staphylococcal or other multi-drug resistant Gram-positive infections (Marsot et al., 2012). It is considered a critically important human antimicrobial by the WHO (WHO, 2018). Similarly to the β–lactams, the mechanism of action is its ability to inhibit cell wall synthesis in bacteria. Vancomycin targets the terminal D-alanyl-D-alanine during the construction of peptidoglycan, which inhibits the building of crosslinks and leaves the cell vulnerable to osmotic pressure (Levine, 2006). Vancomycin is not active against Gram-negative bacteria due to the outer membrane barrier (Walsh et al., 1996). Vancomycin is not used in veterinary medicine in NZ; macrolide antibiotics are instead typically used to treat infections not suitable to be managed with β–lactams (MPI, 2019). Vancomycin-intermediate or resistant *S. aureus* as well as vancomycin-resistant enterococci have been reported in the

literature, with resistance being conferred by gene mutations that allow the building of peptidoglycan to continue with less affinity for vancomycin (Rao et al., 2021).

### 2.4.6 Lincomycin

Lincomycin is a bacteriostatic, narrow-spectrum lincosamide antibiotic that is used to treat Gram-positive infections, with little effect against Gram-negative bacteria (Papich, 2016). Lincomycin is classified as a highly important antimicrobial by WHO (2018). This antibiotic works against bacteria by blocking the synthesis of proteins on the 50S ribosomal subunit by binding to it and causing peptidyltransferase reaction inhibition (Spížek & Řezanka, 2004). Lincomycin, combined with neomycin, is considered an 'orange-light' antibiotic by the NZVA and is indicated for staphylococcal mastitis after penicillin treatment has failed (NZVA, 2018). Resistance to lincomycin is conferred by efflux out of the cell, antibiotic target site modification and inactivation of enzymes such as rRNA methylases (Lüthje & Schwarz, 2007). Lincomycin is notable for its role as an environmental residual; as it is not fully metabolised by cows, it is discarded through effluent and detected in soils and waterways (Mehrtens et al., 2021).

### 2.4.7 Fluoroquinolones

Fluoroquinolones are a class of broad-spectrum antibiotics which include levofloxacin and ciprofloxacin (Mahoney & Swords, 2021). Fluoroquinolones' mechanism of action is inhibition of type II DNA gyrases which are necessary for transcription of mRNA and DNA replication (Grobbel et al., 2007). Fluoroquinolone use in NZ dairy cattle is relatively low and is considered a 'red light' antibiotic, meaning they are only used in hard-to-treat infections where their use is closely monitored by veterinarians (MPI, 2020; NZVA, 2018). The fluoroquinolone antibiotics marbofloxacin and enrofloxacin are indicated for use in coliform mastitis (NZVA, 2018). Resistance to fluoroquinolones is due to four mechanisms: mutations in gyrase or topoisomerase which reduce binding, active efflux or reduced influx, protection of the target enzyme, and antibiotic modifications by bacterial enzymes (Maris et al., 2021).

## 2.5 Antibiotic Susceptibility Testing

With the rise of AMR, accurate methods to test antibiotic susceptibility are more important than ever (Markelz et al., 2011). The Kirby-Bauer disc diffusion assay for antimicrobial susceptibility is considered a standardised procedure for the testing of aerobic and facultative anaerobic pathogenic bacteria (Hudzicki, 2009). The general purpose of a disc diffusion assay in a diagnostic setting is to assist in selecting the most effective antimicrobial treatment for patients (Christenson et al., 2018). In the antibiotic disc diffusion assay, filter paper discs impregnated with a known dosage of antibiotic are placed onto the surface of an agar plate

which has been inoculated by swabbing with a bacterial suspension; after 16–20 hours of incubation in the appropriate environment, plates are read by measuring the zones of inhibition around the antibiotic discs (European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2021). Zone diameters are compared to breakpoint tables, then results are reported as susceptible, intermediate, or resistant (Fekete et al., 1994). The designation of breakpoint data is a consensus process, whereby breakpoints are established by review of literature and clinical data using the most current information available (Clinical and Laboratory Standards Institute [CLSI], 2020).

Antibiotic susceptibility testing can also include minimum inhibitory concentration (MIC) assays. In this assay, a range of antibiotic concentrations are used to determine the lowest effective dosage that visibly inhibits bacterial growth (Belanger & Hancock, 2021). This type of assay can be important when deciding how to treat a bacterial infection due to the selective pressure and resulting antibiotic resistance that arises from inappropriate antibiotic dosing (Geisinger & Isberg, 2017). In both disc diffusion and MIC assays, Mueller-Hinton agar or broth is typically used due to its nutrient-rich nature; however, *in vivo* conditions can be quite different based on the body site of infection therefore *in vitro* results gained using Mueller-Hinton may not be reflective of antibiotic activity during treatment (Belanger & Hancock, 2021). Like with disc diffusion assays, MIC breakpoint data are used for the labelling of bacteria as susceptible, intermediate, or resistant. It should be noted that major discrepancies between official breakpoint values have been observed, which should be taken into consideration when testing (Diene et al., 2017).

## 2.6 Bioinformatics

Bioinformatics is the interdisciplinary application of computation and analysis to the management and interpretation of biological data (Bayat, 2002). It is important in genomics research, as the datasets that arise from this work are increasingly large and impractical for manual analysis. Bioinformatics uses different methods and tools, primarily computer software or sequential algorithms known as ‘pipelines’, to rapidly process information (Ray et al., 2021). The work of bioinformaticians has been invaluable in understanding infectious disease, which includes the spread of AMR (Bah et al., 2018). Bioinformatics tools used for the investigation of AMR in this research include rapid genome annotation software pipeline Rapid Annotation using Subsystem Technology (RAST) (Overbeek et al., 2014) and ARG identifier The Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca) (Alcock et al., 2020)

### 2.6.1 Genome Annotation

Genome annotation is the process of analysing a genome sequence using databases to identify functional features in said genome (Richardson & Watson, 2013). Gene annotation is necessary to retrieve useful information from DNA sequences therefore understand the organism; this information can be used to study its evolution (Ejigu & Jung, 2020). The use of automated functional annotation pipelines such as RAST or Prokka (Seemann, 2014) enables a set of algorithms to process large amounts of sequence data from bacterial and archaeal genomes in a comprehensive and efficient manner that incorporates a multitude of software and databases (Ejigu & Jung, 2020). RAST utilises a populated 'subsystem' approach whereby expertly curated annotation data kept in sets of logically related functional roles (subsystems) are projected onto uploaded genomes (Brettin et al., 2015). Functional roles are assigned to the CDS either by recognition of the CDS within the curated subsystems or evidence from other tools, such as gene prediction software GLIMMER3, which have been integrated into the pipeline (Ejigu & Jung, 2020). Though RAST is generally considered to be highly accurate and consistent, a general criticism is that a default, pre-ordained pipeline may not always be appropriate for all genomes; the recent implementation of the RAST toolkit has modulated the pipeline to allow for a more customisable experience which seeks to address this issue (Brettin et al., 2015).

### 2.6.2 Antibiotic Resistance Gene Identification

The identification of AMR genes is crucial for tracing the epidemiological path of antibiotic resistance (Zankari et al., 2012). There are several antimicrobial resistance databases that hold sequence data for known AMR determinants, which typically work in a similar way; sequences of interest are compared to resistance genes in the BLAST database, which runs a pairwise DNA or protein sequence alignment to find any notable AMR genes (Adu-Oppong et al., 2017). CARD is an actively human-curated database populated with ARG sequences with ongoing updates as new genes are found (McArthur et al., 2013). To be included in the database, sequences must be submitted to GenBank and be associated with peer-reviewed articles, as well as show experimental evidence of minimum inhibitory concentration testing (Alcock et al., 2020; McArthur et al., 2013).

Within CARD, the Resistance Gene Identifier (RGI) tool (https://card.mcmaster.ca/analyze/rgi) performs resistome gene prediction and *de novo* annotation for molecular sequences using protein homology and SNP protein variant models (McArthur & Tsang, 2017; McArthur et al., 2013). RGI operates on the concept of perfect/strict/loose hits; perfect hits are a 100% match to a database reference, strict hits have some variation from the reference sequence to catch

unknown variants or mutations, and loose hits are those beyond the cut-off for strict hits but with distant homology to known AMR genes (Alcock et al., 2020).

## 2.7 *Streptococcus uberis*

*Streptococcus uberis* is a Gram-positive, catalase-negative, cocci-shaped bacterium with chain arrangement of the Streptococcaceae family (Haenni et al., 2018). This bacterium has a genome size of 1.8–2.3 Mb and a guanine-cytosine (GC) content of 35.5-36.5% (Vélez et al., 2017). It is well known for having an extremely high genetic diversity, with both virulent and avirulent strains (Günther et al., 2016). It is commensal at multiple body sites of healthy dairy cattle, and is also commonly found on the farm as an environmental pathogen (Ward et al., 2009). Though *S. uberis* can be considered a contagious pathogen, infection in cattle is mostly a result of environmental transmission (Coffey et al., 2006). Environmental reservoirs include manure, bedding, and pastures (Oliver et al., 2011). Therefore, cattle are consistently exposed to *S. uberis* year-round.

Internationally and in NZ, *S. uberis* is one of the most common causes of both clinical and subclinical bovine mastitis (Coffey et al., 2006; Douglas et al., 2000; McDougall et al., 2014). Due to its role as an environmental pathogen, steps taken to control the proliferation of *S. uberis* such as post-teat disinfection have had little effect on prevention and control of infection (Douglas et al., 2000). Virulence factors for *S. uberis* are not well described, which also creates a barrier for prevention of infection (Oliver et al., 2011). Previous studies that have explored the genetic differences between virulent and avirulent strains have no clear conclusions, therefore it is suggested that host-pathogen interaction is a strong determinant in *S. uberis* intramammary infection (Günther et al., 2016). A compound called *S. uberis* adhesion molecule has been identified by researchers as being involved in the infection of bovine mammary gland epithelial cells (Oliver et al., 2011). Antibiotic treatment is the predominant strategy for clinical mastitis, with penicillin usually being the first choice of antibiotic (Martins et al., 2021). Resistance to erythromycin, tetracycline (Thomas et al., 2015) and trimethoprim-sulfamethoxazole (McDougall et al., 2014) by *S. uberis* has been observed in previous studies. Vaccines are in development (Collado et al., 2018), however, due to the heterogeneity of *S. uberis* strains and lack of knowledge on their virulence factors this has been a difficult task (Douglas et al., 2000; Hossain et al., 2015).

## 2.8 Summary of literature review

This literature review has given an overview of the different aspects of AMR and more, specifically, antibiotic resistance especially within animal husbandry. It has covered general antibiotic use on NZ dairy farms and the pathogens responsible for common dairy cattle

infections. A broad outline of the state of antibiotic use and AMR in NZ has been given, in addition to the origins of and potential solutions to AMR. A brief description of the antibiotics used in this study has also been provided. The purpose of this is to give necessary background for the antibiotic susceptibility testing work performed to answering RQ one (Section 1.2). A summary of the bioinformatics methodology employed in this work has been given, alongside with a description of the role of *S. uberis* as a common mastitis-pathogen. This is provided as background for RQ two (Section 1.2), where the genomic basis for antibiotic resistance in a *S. uberis* isolate is explored. Overall, an emphasis on continued research into AMR into NZ has been made, as understanding the bigger picture of resistance is the key to combating it.

# 3.0 Materials and Methods

## 3.1 Sample Acquisition and Storage

Raw milk samples (A-J) were previously taken from bulk milk tanks on selected dairy farms located in Aotearoa New Zealand by AgResearch Hopkirk (AgResearch, Palmerston North, New Zealand) and stored at -20°C. Aliquots of the raw milk samples in 5 mL screw cap transport tubes were provided by Dr Ali Karkaba and Prof. Scott McDougal (Cognosco, Anexa Vet Services, Morrinsville, New Zealand). Samples were sent to Auckland University of Technology (AUT) in a portable cool box then transferred to -20°C storage.

The MRSA isolate was provided by Dr Sara Burgess (Massey University, Palmerston North, New Zealand). For transportation to AUT, the MRSA isolate was sub-cultured onto Columbia Agar with 5% sheep blood (CBA). The isolate was stored at 4°C.

The quality control *S. aureus* subsp. *aureus* Rosenbach strain ATCC 25923 was supplied by the Laboratory officer for the AUT School of Science microbiology teaching laboratory. The isolate was maintained on cryobeads in -80°C storage, then sub-cultured onto CBA for experimental use. The isolate was stored at 4°C.

## 3.2 Isolate Culturing and Storage

Raw milk samples (A-J) were defrosted at room temperature, agitated, then streaked onto nutrient agar (NA), Baird Parker agar (BPA), and Columbia Agar with 5% sheep blood with esculin (Fort Richard Laboratories Ltd, New Zealand) using the five-phase streak method. Nutrient agar was prepared according to the manufacturer's directions, as follows: 13.8 g of NA powder was suspended in 600 mL of deionised water, then mixture was heated until dissolution was complete, followed by autoclaving at 121°C for 30 minutes under 100 kPa. Once inoculated, plates were incubated at 35°C under aerobic conditions for 24 hours.

Inoculated plates were observed for differential colony morphology. For NA plates, unique colonies were sub-cultured onto fresh NA plates. For BPA plates, black colonies were presumed to be *S. aureus* and were sub-cultured onto fresh NA plates. For Columbia Agar with 5% sheep blood with esculin, black colonies were presumed to be *Streptococcus* spp. and were sub-cultured onto fresh NA plates. All plates were incubated at 35°C under aerobic conditions for 24 hours. Plates were observed for pure, isolated cultures.

In order to create glycerol stocks of isolates, a single colony was sub-cultured to nutrient broth (NB) (Fort Richard Laboratories Ltd, New Zealand) and incubated at 35°C under aerobic conditions for 24 hours. A 25% volume per volume glycerol stock was made with 0.75 mL NB

sub-culture and 0.75 mL 50% glycerol in a 1.5 mL Eppendorf tube. Each isolate was used to make two glycerol stocks, each stored at -20°C and -80°C respectively.

## 3.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out using the Kirby-Bauer antibiotic disc diffusion assay method. The antibiotics and the dosage used for susceptibility testing are listed in Table 1. Isolates were prepared by inoculating 5 mL NB with 0.3 mL of glycerol stock then incubated at 35°C under aerobic conditions for 24 hours. Broths were observed for turbidity then 0.1 mL was applied via spread plating to Columbia Agar with 5% sheep blood (CBA) (Fort Richard Laboratories Ltd, New Zealand). Antibiotic discs (Fort Richard Laboratories Ltd, New Zealand) were placed on each plate along with a blank disc acting as the negative control (Figure 1). Due to the interruption of laboratory research because of the lockdown restrictions associated with the COVID-19 pandemic, not all isolates were able to be tested against Novobiocin. Plates were incubated at 35°C under aerobic conditions for 24 hours.

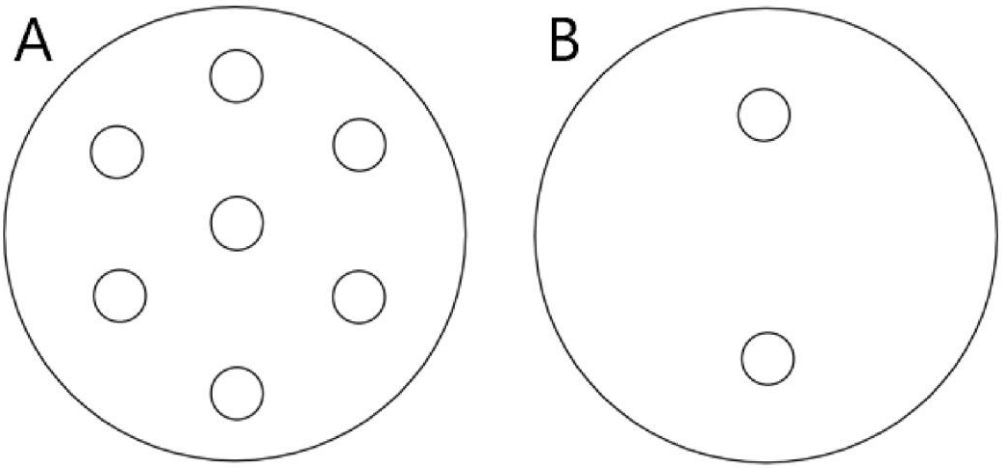


**Figure 1.** Antibiotic disc assay placement for testing of penicillin G, ampicillin, amoxicillin + clavulanic acid, neomycin, vancomycin, and cefuroxime (A) and novobiocin-only testing (B).

Plates were observed for zones of inhibition around the antimicrobial discs. Zones were measured as the diameter of the zone across the centre of the disc, as well as distance from edge of disc to the edge of the zone of inhibition (Figure 2). The boundary of the zone was judged by a general clearing of bacterial isolates; a few isolated colonies within the zone were dismissed as outliers. Each isolate was tested against each antibiotic once.

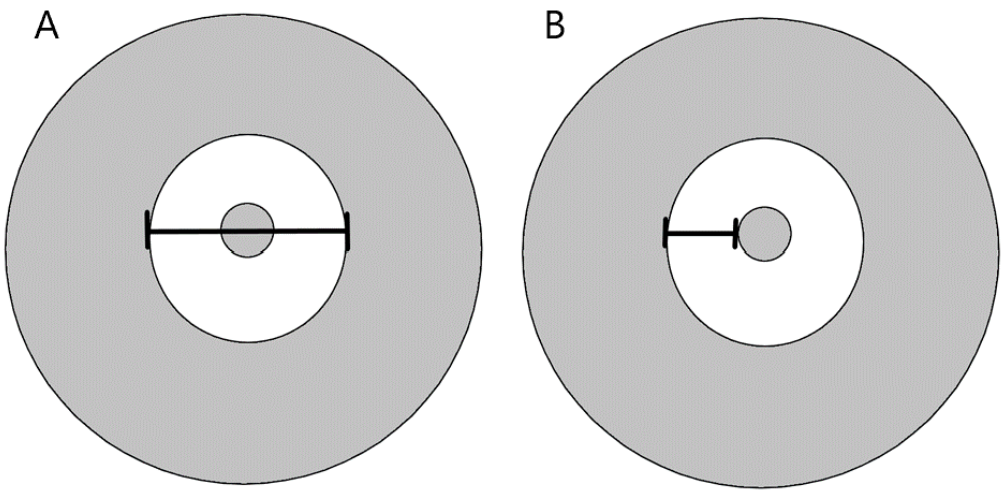


**Figure 2.** Zone of inhibition diagram showing measurement for diameter of the zone across the centre of the disc (left) and distance from edge of disc to the edge of the zone of inhibition (right)

As the milk sample isolates were only presumptively identified and access to current clinical standards for antibiotic resistance were not available, resistance was judged using a mixture of data for the Control *S. aureus,* older clinical standard guides, and selected literature. According to clinical standards, if uncertainty of susceptibility category occurred, the result was downgraded from susceptible to intermediate, intermediate to resistant, or susceptible to resistant (EUCAST, 2021). The criteria for determination of resistance are given in Table 1.

**Table 1.** Antibiotic and relative concentration (µg) plus zone of inhibition diameter (mm) interpretive criteria for determination of resistance status by antibiotic disc diffusion assay

| Antibiotic + Presumptive bacterial genus/species | Concentration (µg) | Zone diameter breakpoints (mm) | | |
|---|---|---|---|---|
| | | Susceptible | Intermediate | Resistant |
| Penicillin G | 2 | | | |
| *S. aureus*[1] | | ≥29 | - | ≤28 |
| Enterococci/Group D Streptococci[1] | | ≥15 | - | ≤14 |
| Ampicillin | 10 | | | |
| *S. aureus*[2] | | ≥27 | - | ≤26 |
| Enterococci/Group D Streptococci[1] | | ≥17 | - | ≤16 |
| Amoxicillin + clavulanic acid | 15 | | | |
| *S. aureus*[2] | | ≥20 | - | ≤19 |
| Enterococci/Group D Streptococci[2] | | ≥10 | 9 | ≤8 |
| Cefuroxime | 30 | | | |
| *S. aureus*[2] | | ≥23 | 15−22 | ≤14 |
| Enterococci/Group D Streptococci[1] | | ≥26 | - | ≤25 |
| Neomycin | 30 | | | |
| *S. aureus*[3] | | ≥17 | 13−16 | ≤12 |
| Enterococci/Group D Streptococci[4] | | ≥20 | 17-19 | ≤16 |
| Novobiocin | 5 | | | |
| *S. aureus*[4] | | ≥14 | - | ≤13 |
| Enterococci/Group D Streptococci[4] | | ≥13 | 11−12 | ≤10 |
| Vancomycin | 30 | | | |
| *S. aureus*[1] | | ≥15 | - | No zone |
| Enterococci/Group D Streptococci[1] | | ≥17 | 15−16 | ≤14 |

Numeral superscript denotes source of zone of inhibition breakpoint data; [1] = CLSI, 2020 [2] = EUCAST, 2021, [3] = Sarker et al., 2014 [4] = Rosco Diagnostica, 2013

## 3.4 Commercial Kit DNA Extraction

DNA was extracted from MRSA using a commercial DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) per the manufacturer's instructions for Gram positive organisms (Qiagen, 2020). Two extractions from the same isolate were performed simultaneously using the same methods. The isolate was prepared by inoculation of 5 mL of NB with 0.3 mL of glycerol stock then incubated at 35°C under shaking conditions for 15 hours. A 1.5 mL NB cell suspension was transferred to a microcentrifuge tube and centrifuged for 10 minutes at 5000 x *g* at 20°C. After discarding the supernatant, the bacterial pellet was resuspended in 180 µL enzymatic lysis buffer. The cells were lysed using 10 mg/mL Lysozyme from chicken egg white (Sigma-Aldrich, Missouri, USA) for 45 minutes at 37°C with an Allsheng Incubator MiniT-100 heating block (Zhejiang, China). After addition of 25 µL Proteinase K and 200 µL Buffer AL (without ethanol), the sample was vortexed then incubated at 56°C for 30 min. The sample was vortexed with 200 µl of 100% ethanol, transferred into a DNeasy Mini spin column inserted in a 2 mL collection tube, then centrifuged at 6000 x *g* for 1 minute at 20°C. Any flow-through was discarded. The spin column was inserted into a fresh collection tube and 500 µL Buffer AW1 was added before being centrifuged at 6000 x *g* for 1 minute at 20°C. Any flow-through was

again discarded. The spin column was transferred into a fresh collection tube and 500 µL Buffer AW2 was added before centrifuging for 3 min at 20,000 x *g* at 20°C. After discarding the flow-through the spin column was placed in a 2mL microcentrifuge tube, then 50 µL of Buffer AE was pipetted directly onto the membrane of the column. The column was incubated at room temperature for 1 minute, and then followed by centrifugation at 6000 x *g* for 1 minute at 20°C. Addition of 50 µL of Buffer AE, incubation and centrifugation was repeated once more into the same microcentrifuge tube to complete the full elution step. The tubes of extracted DNA were transferred to a -20°C freezer for storage.

Stock concentration of extracted DNA was measured using the Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Massachusetts, USA) per the manufacturer's instructions using an Invitrogen™ Qubit™ dsDNA High Sensitivity Assay Kit (ThermoFisher Scientific, Massachusetts, USA).

Quality and contamination of DNA was measured using a GE NanoVue spectrophotometer (GE Biosciences, New Jersey, USA). The $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios were recorded per the manufacturer's instructions, with 2 µL Milli-Q water used for standardisation.

## 3.5 Agarose Gel Electrophoresis

To confirm successful DNA extraction, a 1% agarose, 1 X Tris-Borate ethylenediaminetetraacetic acid
(TBE) gel with 2.5 µL of 10 mg/mL redsafe was run at 85 V for 50 minutes. A 1000 bp molecular weight marker (Sigma Aldrich, Missouri, USA) was used, which was added to 1 µL loading dye and 4 µL Milli-Q water. Each DNA sample lane contained 1 µL loading dye with 5 µL of DNA. Gel results were imaged using an AlphaImager® HP (Alpha Innotech, California, USA) under a ultraviolet transilluminating Ethidium Bromide filter.

## 3.6 Genomic Analysis

### 3.6.1 Genome Acquisition

Due to the interruption of wet laboratory research because of the lockdown restrictions associated with the COVID-19 pandemic, genomic analysis of the extracted DNA could not be carried out. In place of this, 24 assemblies and primary read data of *S. uberis* genomes were provided for analysis by Professor Gregory M. Cook of the Department of Microbiology and Immunology at the University of Otago, and George Taiaroa of The Peter Doherty Institute for Infection and Immunity at The University of Melbourne.

The *S. uberis* isolates were collected between 2006 and 2010 from New Zealand dairy cows with clinical and sub-clinical mastitis infections. The isolates were a mixture of from collaborating veterinary professionals and academics

Genomic DNA was prepared for *S. uberis* isolates from a single colony using a QIAsymphony DSP DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Library preparation was performed with Illumina Nextera XT DNA library chemistries with 150 bp paired end reads and sequenced on a NextSeq 500 (Illumina, CA, USA).

### 3.6.2 Antibiotic Resistance Genes Identification

To identify any potential ARGs present in the *S. uberis* genomes, all 24 assemblies were submitted to CARD's RGI online portal (https://card.mcmaster.ca). The inclusion criteria for each search were set to perfect and strict hits, including a nudge at ≥95% identity loose hits to strict. The genome that returned the highest number of hits in this range, referred to as genome 14, was submitted again to locate perfect, strict, and loose hits. This was performed to identify other genes that may be associated with perfect or strict hits, or antibiotic classes that the perfect and strict hits may confer resistance to.

Genome 14 was uploaded to the RAST v2.0 server (http://rast.nmpdr.org/) to produce gene annotations and predictions. Genes flagged in the 'resistance to antibiotics and toxic compounds' category using the SEED Viewer were noted and cross-referenced with the CARD hits output to identify common hits.

### 3.6.3 Comparative Genomics

All 24 *S. uberis* genome assemblies, including the RAST-annotated genome 14, were uploaded to the software programme Geneious Prime v2021.2.2 (https://www.geneious.com) for gene visualisation and as a CDS source for BLAST searches.

The BLASTn programme (https://blast.ncbi.nlm.nih.gov/) was used to identify sequence similarity between potential ARGs identified by CARD plus transposases in genome 14 and reference nucleotide sequences. Reference sequences were located via literature which had confirmed ARGs or transposases. For investigation of the *lnu*(C) gene and associated transposase, an *S. uberis* genome originating from a dairy cow with clinical mastitis in Queensland, Australia (accession number: GCA_016837545.1) was used (Vezina et al., 2021). For investigation of *patA*/*patB,* no *S. uberis* with these confirmed genes could be located therefore *Streptococcus pneumoniae* strain R6 originating from a clinical isolate (accession number: GCA_000007045.1) was used (Boncoeur et al., 2012; Hoskins et al., 2001; Marrer et al., 2006). A 'highly similar' sequence search was selected in the first instance for each BLAST

query. This was followed by a 'somewhat similar' query if no suitable highly similar result was returned in order to capture the same ARGs in other *Streptococcus* species. For 'somewhat similar' results, only the top hit was investigated for further analysis.

The IS Finder database (https://isfinder.biotoul.fr/) was utilised in conjunction with the inbuilt BLASTn tool to identify the IS family and group of the putative *lnuC*-associated transposase located in genome 14. The query sequence was submitted to the IS finder database with pairwise alignment view selected.

# 4.0 Results and Discussion

## 4.1 Antibiotic Susceptibility Testing

22 isolates from raw milk were tested against select antibiotics for susceptibility using the Kirby-Bauer disc diffusion method. An example of the results of a disc diffusion assay is given in Figure 3. Isolates were presumptively identified by genus according to what selective media they grew on. However, species was not presumed nor confirmed. For simplicity, the presumptive *Staphylococcus* spp. isolates were measured against *S. aureus* breakpoint criteria and isolates of *Streptococcus* and *Enterococcus* spp. against the average breakpoint values of *Enterococcus faecalis* and *S. pneumoniae*.

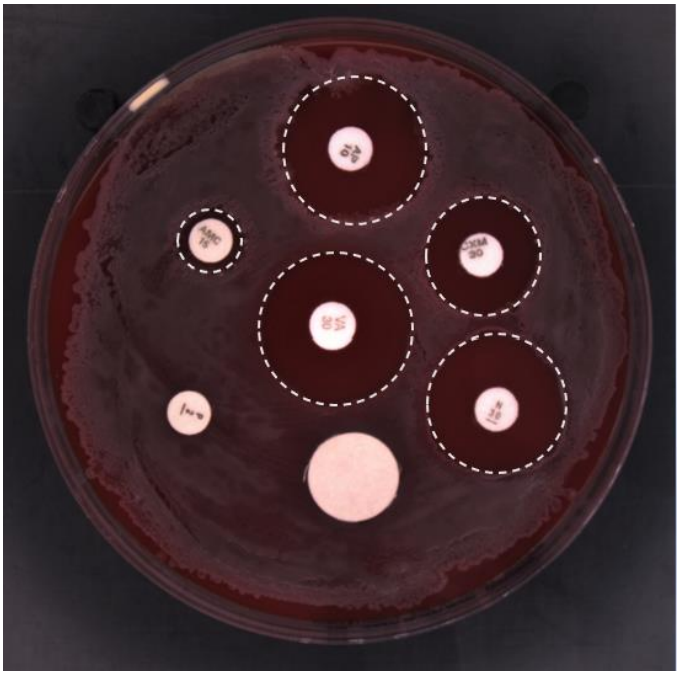

**Figure 3.** An example of the Kirby-Bauer disc diffusion method on CBA. Dashed circles display the boundary of the zone of inhibition. Plate shown is isolate MSEBE.3.

### 4.1.1 Presumptive *Staphylococcus* spp.

Isolates were determined to be presumptive staphylococci by the growth of grey to black colonies on BPA. For the presumptive *Staphylococcus* spp. grown on BPA, all isolates were resistant to penicillin G (Figure 4). None were resistant to neomycin, with all isolates being intermediate or susceptible. Of those tested against novobiocin, the majority were susceptible. Most were resistant to ampicillin, amoxicillin plus clavulanic acid and cefuroxime. Results for vancomycin were varied, with a similar number of isolates being categorised as resistant, susceptible or inconclusive.

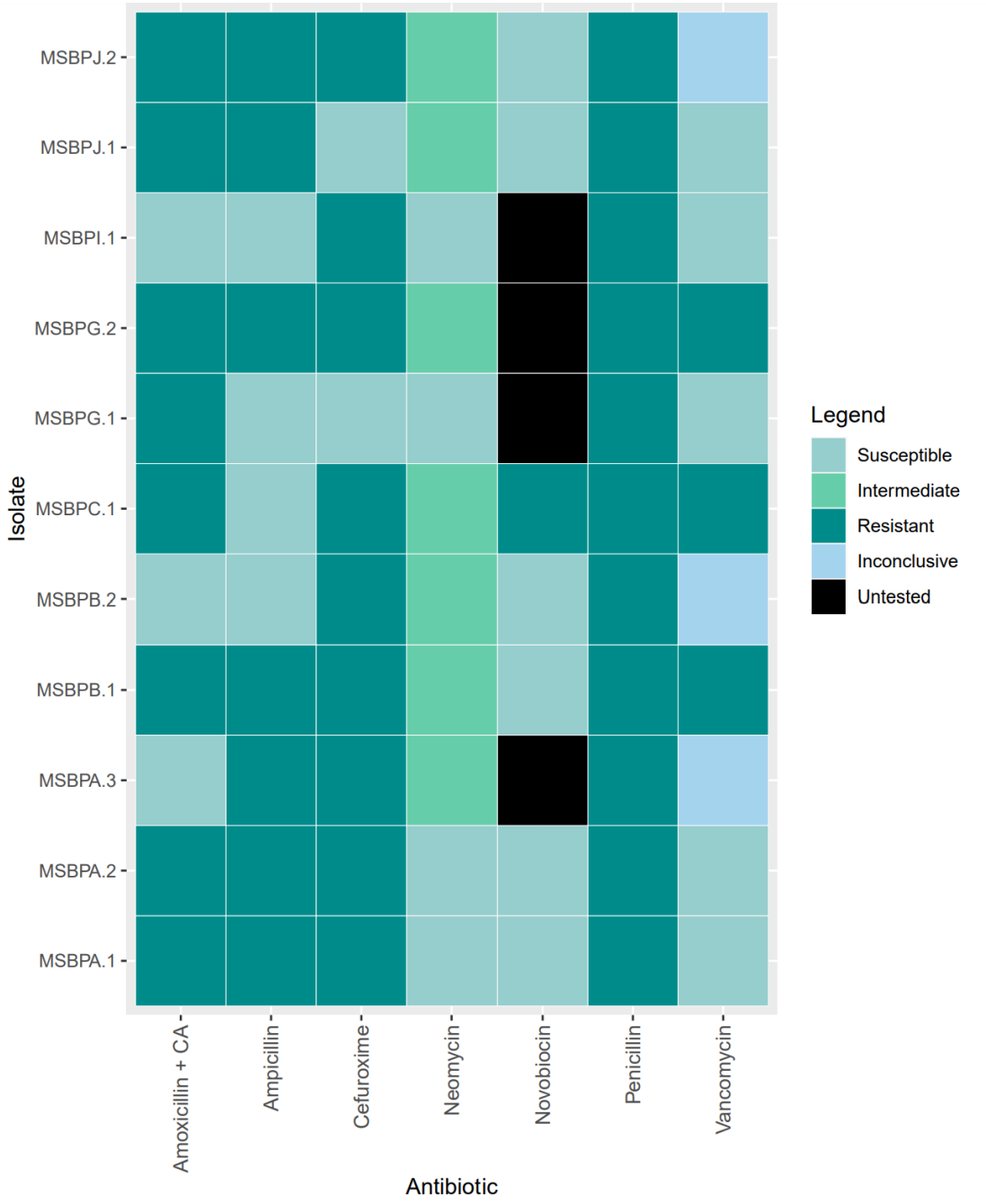


**Figure 4.** Heatmap displaying designation of susceptibility from antibiotic susceptibility testing data for presumptive *Staphylococcus* spp. isolates from BPA. CA = clavulanic acid.

Across the board resistance to penicillin is different from what would be expected from *Staphylococcus* spp.. Though Penicillin-resistance in *S. aureus* has been on a downward trend over the last 40 years, it is still seen in approximately 30–40% of mastitis-associated *S. aureus* in NZ (Greening et al., 2021; McDougall et al., 2014; NZVA, 2018). The penicillin dosage used in this research was 2 µg, which is moderate when compared to dosages in other studies on antibiotic susceptibility disc diffusion; typical dosages range between 0.5–10 µg (BacDive, n.d.; CLSI, 2020; EUCAST, 2021). According to clinical standards, a susceptible *S. aureus* tested

against penicillin G (0.6 µg) isolate should show a zone of inhibition of at least 26 mm, with anything less being considered resistant (CLSI, 2020). It is assumed that the lack of penicillin efficacy was likely due to the age and/or storage conditions of the antibiotics, which is discussed in full below. Additionally, antibiotic susceptibility data in the literature comes from milk from cattle with and without sub-clinical or clinical mastitis which could influence the resistance profiles of the isolates tested (McDougall et al., 2014).

For the other β-lactams—amoxicillin plus clavulanic acid, ampicillin, and cefuroxime—most staphylococcal isolates showed resistance. Again, this is not in line with testing performed in other studies. In a study by McDougall et al. (2014) resistance against amoxicillin plus clavulanic acid was shown to be 0%, and for ampicillin, 27%. Cefuroxime could not be calculated due to a lack of cut-off values, but at the highest dose of the antibiotic used (4 µg/mL), only 0.5% of the tested isolates were not inhibited. A similar study also found 100% susceptibility in NZ and United States *S. aureus* milk isolates against amoxicillin plus clavulanic acid (Petrovski et al., 2015). In another recent NZ study that tested raw milk isolates against cefoxitin (30 µg), a common clinical analogue of cefuroxime, susceptibility was displayed in 50 out of 51 *S. aureus* isolates (Greening et al., 2021). Similar penicillin and ampicillin resistance profiles for staphylococcal isolates would not be unusual due to the similarity in antibiotic activity—the broad-spectrum of ampicillin compared to the narrow-spectrum of penicillin confers additional bactericidal activity toward Gram-negative bacteria, which is irrelevant here (Suleyman & Zervos, 2016). However, in comparison to literature, resistance to amoxicillin plus clavulanic acid seen in this study is unusually high. Again, differences are assumed to be due to lack of potency in the antibiotic discs in addition to the other research limitations discussed below.

The staphylococcal isolates mostly showed a mixture of intermediary and susceptibility to neomycin, which is comparable to literature. Petrovski et al. (2015) found 100% susceptibility to neomycin (30 µg) in their testing of raw milk *S. aureus* isolates, where McDougall et al. (2014) found some resistance at lower dosages which quickly tapered off as dosage increased. The intermediate isolates may have occurred in response to a slight lack of efficacy in the antibiotics, or limitations such as using human clinical breakpoints for veterinary testing which can be responsible for difference in susceptibility categorisation (McDougall et al., 2014).

For simplicity of reporting, a mixture of clinical breakpoints and literature were used to designate categories of susceptibility for the vancomycin testing. However, vancomycin susceptibility in staphylococci cannot be accurately determined using disc diffusion assays (CLSI, 2020; EUCAST, 2021). Instead, MIC tests are used to differentiate resistant, intermediate, and susceptible strains; this is because disc diffusion assay will often return

zones of similar sizes regardless of resistance profile. Disc diffusion can be used to screen an isolate in the first instance to see if MIC tests may need to be performed (Wongthong et al., 2015). Based on the results seen in this work, several of the isolates tested would be suitable candidates for follow-up MIC testing if it had been performed. Vancomycin is not utilised as a veterinary medicine in NZ, however, in several studies vancomycin-resistant *S. aureus* have been found in bovine milk at a rate of 3.4–84.6% of total *S. aureus* isolates (Javed et al., 2021).

### 4.1.2 Presumptive *Enterococcus* spp. and *Streptococcus* spp.

For the presumptive *Enterococcus* spp. and *Streptococcus* spp. isolates from CBA with esculin, less resistance overall was seen in comparison to the presumptive *Staphylococcu*s spp. isolates (Figure 5). All isolates were again resistant to penicillin G. For the other penicillins, amoxicillin plus clavulanic acid and ampicillin, susceptibility is high. In comparison with the *Staphylococcu*s spp. isolates, susceptibility to neomycin was higher. Novobiocin showed mixed results.

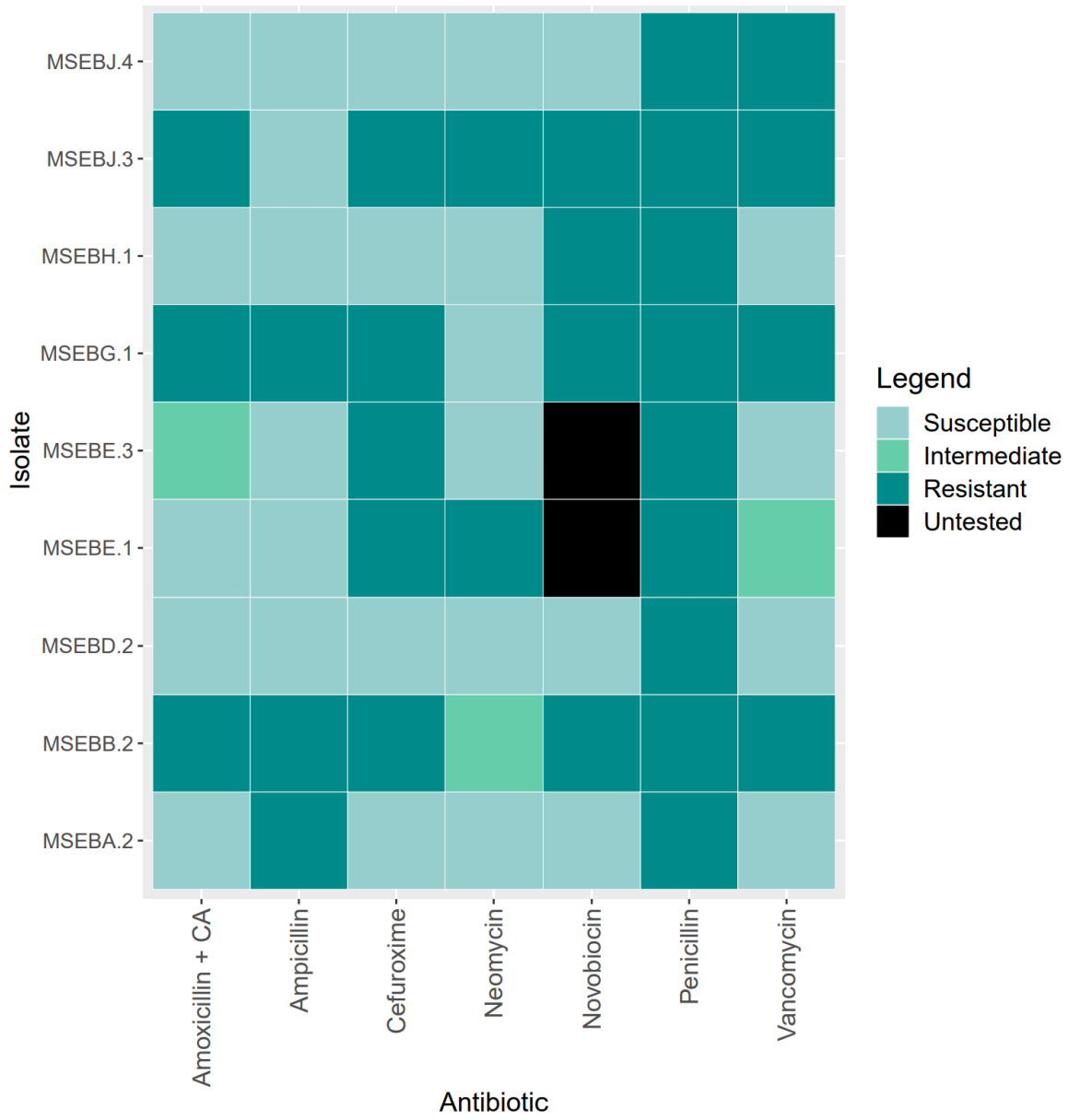


**Figure 5.** Heatmap displaying designation of susceptibility from antibiotic susceptibility testing data for presumptive *Enterococcus* spp. and *Streptococcus* spp. isolates from CBA with esculin. CA = clavulanic acid.

Similarly to the staphylococci, 100% resistance to penicillin G was seen (Figure 5). Penicillin-resistance in mastitis-causing *Streptococcus* spp. has historically been low, though rising MIC doses have been noted in recent years in *S. uberis* (Haenni et al., 2018; McDougall et al., 2020). In similar studies, susceptibility to penicillins in streptococcal isolates was high, with no more than 1% of isolates being categorised as resistant (McDougall et al., 2014; Petrovski et al., 2015). Penicillin resistance in enterococci has also been shown to be uncommon (Lines et al., 2019). This result supports the suspicion that the penicillin used in this study lacked appropriate efficacy.

In comparison to the penicillin results, ampicillin and amoxicillin plus clavulanic acid susceptibility was high, which is in line with previous literature. Many streptococci do not produce β-lactamases, though mutations in the PBP can confer resistance, which means effectiveness remains high (von Specht et al., 2021). Studies have shown that resistance to these two antibiotics is in the range of 1–10%, with rates of resistance to ampicillin being slightly higher (McDougall et al., 2014; Petrovski et al., 2015). In enterococci, ampicillin and amoxicillin plus clavulanic acid-resistance is also typically very low (Maasjost et al., 2015). The rate of resistance in this study was slightly higher, but given the wider possibility of genus and species, it is likely that misclassification based on presumptive testing has occurred.

Similarly to the staphylococci, resistance to vancomycin for *Streptococcus* spp. also cannot be measured purely using disc diffusion but can be screened using this method (CLSI, 2020). However, *Enterococcus* spp. can be assessed with disc diffusion, though all intermediate isolates should be further investigated with MIC testing (CLSI, 2020). As a mix of results was noted for these isolates, including one intermediate categorisation, further MIC tests should be carried out.

Out of the isolates tested, over half were resistant to novobiocin. There is very little literature on novobiocin susceptibility in streptococci or enterococci; this may be in part because it is an older antibiotic that, due to early resistance and issues with side effects, is not widely used (Rodríguez-Cerrato et al., 2010). Resistance to novobiocin is only regularly discussed in regards to the human pathogen *S. pneumoniae*; thus, there does not appear to be any intrinsic resistance that would explain this result (Dupont et al., 2005; Rodríguez-Cerrato et al., 2010). One study that used novobiocin (30 µg) against select bovine mastitis pathogens found 100% susceptibility across the streptococci and enterococci isolated (Thornsberry et al., 1997). However, given that the dosage used in this study was 5 µg, it is possible that the vast difference in potency easily explains the resistance seen in these isolates.

### 4.1.3 Quality Control *S. aureus* and MRSA

For both the quality control *S. aureus* subsp. *aureus* Rosenbach strain ATCC 25923 and MRSA isolates, resistance to penicillin G, cefuroxime, and amoxicillin plus clavulanic acid was displayed (Figure 6). No overt resistance was shown to vancomycin or novobiocin, with neomycin and ampicillin having mixed results.

Methicillin-resistant *S. aureus* are famously resistant to penicillins, and the majority of β-lactam antibiotics (Hamilton & MacGowan, 2019). Thus, resistance to penicillin G, ampicillin, and cefuroxime was the expected result (Figure 6). Resistance to β-lactams in *mecA*-MRSA is conferred by the acquired low-affinity penicillin-binding protein 2A, which is not affected by

the blocks caused by these antibiotics (Guignard et al., 2005). However, in *mecC*-MRSA, resistance is a combination of penicillin-binding protein 2A and the production of a β-lactamase; therefore, treatment with amoxicillin plus clavulanic acid is sometimes successful (Ba et al., 2015). It is possible that the MRSA variant used in this study has the *mecA* genotype.

In this study, MRSA was deemed susceptible to novobiocin. Novobiocin has been shown to work against MRSA, with several studies resulting in at least partial success against the majority of isolates (Sadiq et al., 2020; Standiford et al., 1993; Walsh et al., 1985). However, a study of MRSA in dogs found that it was not susceptible to novobiocin (Fulham et al., 2011). Given that MRSA has multiple strains depending on location and origin, correctly understanding the antibiotic resistance profile may include genomic investigation into specific resistance genes (Turner et al., 2019).

Vancomycin resistance in MRSA has been increasing in recent years, which is of particular concern as it is currently the choice of antibiotic for such infections (Tadesse et al., 2018). Therefore, the inconclusive result seen here would warrant further investigation with MIC testing.

The quality control strain *S. aureus* subsp. *aureus* Rosenbach strain ATCC 25923 was used to assess the efficacy of antibiotics and as a comparison to MRSA. Testing revealed a similar resistance profile to MRSA but had susceptibility to ampicillin and an intermediate reaction to neomycin (Figure 6). Records for this strain show it should have susceptibility toward penicillin G (6 µg), ampicillin (10 µg), cefuroxime (30 µg), vancomycin (30 µg), neomycin (30 µg), and amoxicillin plus clavulanic acid (30 µg) (BacDive, n.d.; EUCAST, 2021). Unfortunately, no strain-specific data could be found for novobiocin. However, literature indicates that it should have bactericidal activity against the quality control strain (Thornsberry et al., 1997). Resistance to penicillin G, cefuroxime, and amoxicillin plus clavulanic acid is inconsistent with what should be seen in *S. aureus* ATCC 25923. There are several suggestions for what may have caused this. Firstly, penicillin in this study was of a lower dosage, which may be subinhibitory for this strain. Secondly, as previously mentioned, there is concern that the antibiotics used did not have full potency which is discussed in the next section. Lastly, antimicrobial resistance has been shown to change overtime when bacteria kept in cold storage for prolonged periods; therefore it is possible that the *S. aureus* ATCC 25923 resistance phenotype has altered (Mayhall & Apollo, 1980). Other limitations of the work performed which may have affected testing are described below in Section 4.1.4.

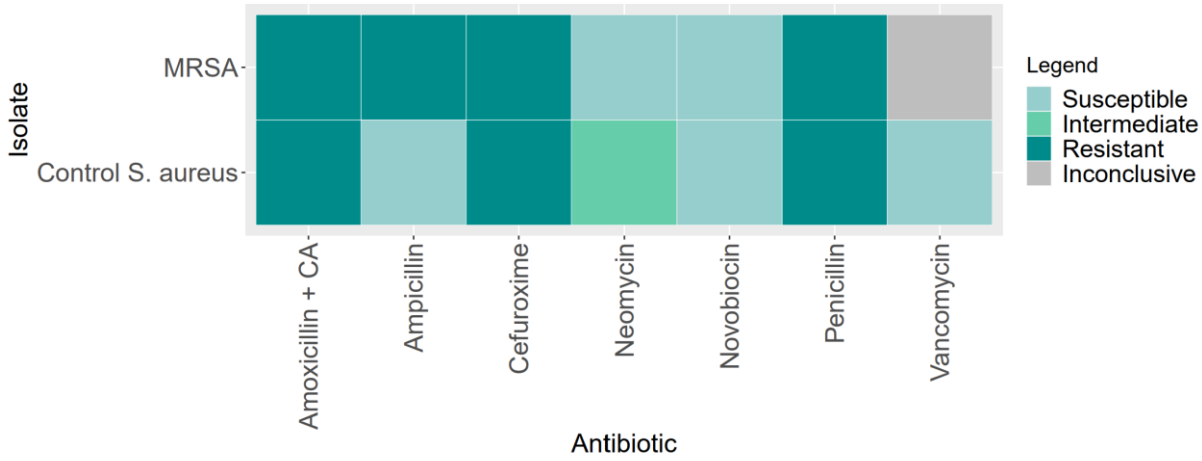


**Figure 6**. Heatmap displaying designation of susceptibility from antibiotic susceptibility testing data for the quality control *S. aureus* subsp. *aureus* Rosenbach strain ATCC 25923 and MRSA isolates. CA = clavulanic acid.

### 4.1.4 Limitations

There were several limitations faced during antibiotic susceptibility testing. The antibiotic discs used in the Kirby-Bauer disc diffusion assay had been kept in cold storage at 4°C for an unknown, prolonged period before commencement of this study. It is known that the efficacy of antibiotics lessens over time; though older antibiotics are unlikely to cause harm, their active compounds can degrade and may not have the same bactericidal or bacteriostatic activity (Ogunshe & Adinmonyema, 2014). Additionally, once packages of antibiotic discs or strips are opened, their expiry is brought forward unless stored with a desiccant under precise temperatures (Bayot & Bragg, 2021). Antibiotics are very sensitive to moisture, and without desiccant or coating can lose potency (Ogunshe & Adinmonyema, 2014). Therefore, it is possible that the antibiotics used did not display the expected activity of the given dosage due to a loss of efficacy, resulting in weakened bactericidal/bacteriostatic activity.

Due to availability CBA was used as the solid media to perform antibiotic susceptibility testing on. Though solid media is standard for the Kirby-Bauer disc diffusion assay method, Mueller-Hinton agar (MHA) is typically used due to the lack of inhibitors in the agar and supports the growth of most nonfastidious bacterial pathogens (Hudzicki, 2009). Additionally, as this has become the standard agar, most breakpoint data for susceptibility is collected from tests using MHA; as such, any testing done with a different medium is not directly comparable (CLSI, 2020; EUCAST, 2021; Hudzicki, 2009). There are no direct comparisons of CBA and MHA in the literature, however, there are several studies which investigate the discrepancies between

MHA and other agars (Brenner & Sherris, 1972; Russell et al., 2006). Additionally, quality control strains are also typically tested using MHA (Hakanen et al., 2002). Thus, zone of inhibition measurement and designations of susceptibility are only somewhat comparable to official breakpoint data.

Due to time and budget constraints, MIC testing was not performed. Testing for MIC can be useful for verification of disc diffusion assay results, as well as giving more precise dosage data and degree od susceptibility in clinical settings (Kowalska-Krochmal & Dudek-Wicher, 2021). Additionally, for some combinations of bacteria and antibiotic, a quantitative MIC assay is more accurate due to issues like poor antibiotic penetration into solid medium (Kowalska-Krochmal & Dudek-Wicher, 2021). For vancomycin, MIC testing is necessary to correctly assess susceptibility; disc diffusion can only indicate the necessity to complete MIC testing (CLSI, 2020; EUCAST, 2021). It is possible that by carrying out MIC testing with fresh antibiotic solution, that the aforementioned issues with unknown antibiotic potency may have been negated.

As previously mentioned, species for isolates were not known. Breakpoint data are sometimes species-specific, and at other times genus-specific. The clinical standards used for this research differentiate between clinically-important *Staphylococcus* spp. and the different Lancefield groups for *Streptococcus,* but are generalised for *Enterococcus* (CLSI, 2020; EUCAST, 2021). As such, designations of susceptibility using the official breakpoint data are approximate according to the assumptions of what the isolates were likely to be.

## 4.2 DNA Extraction Quality and Quantity

A Qubit® Fluorometer was used to measure the quantity of DNA extracted from the MRSA isolate, and a NanoVue spectrophotometer was used to measure the quality. The results are displayed in Table 2.

**Table 2.** Qubit® Fluorometer and NanoVue spectrophotometer results for quantity and quality of extracted MRSA DNA using a commercial kit

| Extraction | Total DNA quantity (ng) | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
|---|---|---|---|
| MRSA 1 | 736.96 | 1.848 | 0.292 |
| MRSA 2 | 780.2 | 1.217 | 0.791 |

For the quantity of DNA extracted from the MRSA isolate, the MRSA 2 extraction yielded a higher quantity at 780.2 ng. Yet, both extractions were considered a moderate yield for a

commercial kit *S. aureus* extraction. The yield is comparable to other MRSA and *S. aureus* commercial kit DNA extractions, with values between 486–836 ng seen in the literature (Lara et al., 2018; Paul et al., 2014). However, this would likely be too low a yield to use for high-quality whole genome sequencing (Psifidi et al., 2015).

Commercial DNA extraction kits are valuable due to their relative speed and lack of contamination in comparison to other popular methods such as phenol-chloroform DNA extraction, but are known to have a highly variable level of recovered DNA (Hassanzadeh et al., 2016). In comparison, cetyltrimethylammonium bromide extraction can also be highly variable with low reproducibility, and has higher chance of contamination due to differences in how reagents are added (Willner et al., 2012). The use of bead beating, a mechanical process which disrupts the bacterial cell wall, has been shown to increase *S. aureus* DNA yield from mastitis-infected cow milk when compared to standard commercial kit extraction (Unno et al., 2015). Mechanical lysis has generally been demonstrated as the most efficient way to retrieve a high yield of DNA from hard-to-lyse Gram-positive bacteria (Willner et al., 2012).

Like all Gram-positive organisms, the bacterium *S. aureus* has a thick peptidoglycan layer within its cell wall which can make extraction less effective (Lara et al., 2018). Additionally, *S. aureus* is particularly difficult to lyse as its peptidoglycan layer is lysozyme hydrolysis resistant which is due to the O-acetylation of peptidoglycan by O-acetyl transferase (Pushkaran et al., 2015). Lysostaphin, an *S. aureus* specific bacteriolytic enzyme, is typically recommended instead of lysozyme for DNA extraction (Cho et al., 2021; Qiagen, 2020). In this study, lysozyme was used due to issues of availability which is likely to have strongly contributed to low DNA yield.

The initial number of MRSA cells in the culture solution used in the extraction was not known, so a direct comparison is difficult. Therefore, it is possible that a low DNA quantity is partially owed to a low initial CFU.

The measures for the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios for both extractions were mixed in quality (Table 2). For isolate MRSA 1, the $A_{260}/A_{280}$ ratio is 1.848 which is considered above the acceptable threshold for DNA purity without erroneous proteins or extraction reagents (Desjardins & Conklin, 2010). Both MRSA 1 and 2 had poor $A_{260}/A_{230}$ ratios, with MRSA 1 having a substantially smaller ratio of 0.292. An optimum $A_{260}/A_{230}$ ratio for DNA is 2.0–2.2, therefore both extractions fall short of the minimum for this measure of purity (Koetsier & Cantor, 2019).

The MRSA 2 $A_{260}/A_{280}$ ratio of 1.217 suggests protein or reagent contamination of the extracted DNA. Protein contamination can occur if the Proteinase K digestion step was not

carried out to completion (Koetsier & Cantor, 2019). A known issue of using commercial kits is the sometimes-low purity of DNA extracted, which can be due to the carryover of buffers after centrifugation when performing commercial silica-column extractions (Hassanzadeh et al., 2016; Jue et al., 2020). This can be improved by including a two-phase wash step prior to elution (Jue et al., 2020).

The $A_{260}/A_{230}$ ratio represents purity of DNA regarding the presence of unwanted organic compounds, such as residual guanidine, which can be an issue in column based commercial kits (Desjardins & Conklin, 2010). Low $A_{260}/A_{230}$ can be also caused by having low amounts of DNA, as happened in this research, which may explain why both values were quite low (Koetsier & Cantor, 2019). Low $A_{260}/A_{230}$ ratios can often be improved by optimising the extraction process; improving both the DNA yield and eluting the DNA solution more efficiently would likely improve this measure (Desjardins & Conklin, 2010).

Though both extractions were subject to the same conditions and carried out simultaneously, as each extraction is still performed individually it is possible slight differences in technique during the elution process decreased the purity of the extractions. Improving the DNA extraction by using lysostaphin or mechanical lysing, and performing a washing step on the silica column would likely optimise these ratios.

## 4.3 Agarose Gel Electrophoresis

The results of the agarose gel electrophoresis for the DNA extraction of the MRSA isolate is shown in Figure 7. The gel displays a defined band for both MRSA extractions above the 10,000 bp marker. The clarity of bands is moderate, with some smearing seen below the DNA band. This suggests a lack of RNA contamination but a small amount of protein contamination, which is consistent with the $A_{260}/A_{280}$ ratio results in Table 2 (Section 8.2). Neither of the MRSA bands are bright in appearance, suggesting lower amounts of DNA which is supported by the Qubit® Fluorometer results in Table 2 (Section 8.2).

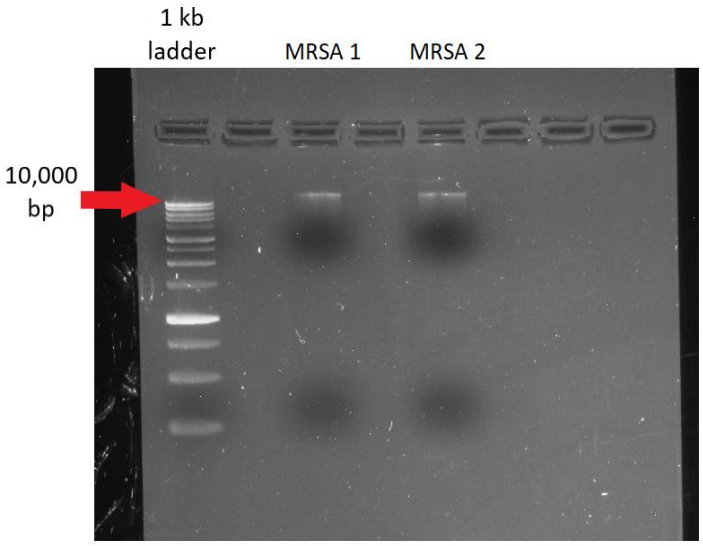


**Figure 7.** 1% agarose TBE gel for confirmation of successful DNA extraction from MRSA isolate using the Qiagen DNeasy Blood and Tissue kit. Lane 1: 1kb DNA ladder, Lane 3: First DNA extraction from MRSA isolate, Lane 5: Second DNA extraction from MRSA isolate.

## 4.4 Genomic Features

As lockdowns associated with COVID-19 prevented the DNA sequencing of isolates used in antibiotic susceptibility testing, it was not possible to investigate the genomic features of any isolates that displayed antibiotic resistance. To circumvent this, assemblies of mastitis-associated *S. uberis* isolates that had already been sequenced were provided for genomic analysis. All isolates were run through CARD for initial identification of ARG. Out of 24 isolates, genome 14 showed the highest number of strict CARD hits. Therefore, genome 14 was chosen as the focus of this analysis.

The genomic features for *S. uberis* Genome 14 given by RAST are displayed in Table 3. The genome size is 1,873,945 bp, with a GC content of 36.4%. When comparing this output to *S. uberis* complete genomes submitted to GenBank, Genome 14 seems equivalent to a complete genome sequence (Taiaroa et al., 2018; Vélez et al., 2017; Vezina et al., 2021; Ward et al., 2009). Genome size and GC content range of *S. uberis* is has been measured at 1.8–2.3 Mb and 35.5-36.5%, respectively (Vélez et al., 2017). The only notable difference is that of total RNA genes. The RAST output puts Genome 14 at 49 total RNA genes, whereas other annotations give numbers of 78–92 (Taiaroa et al., 2018; Vezina et al., 2021). The RAST webpage interface does not provide a breakdown of subset RNA biotypes, so it is unknown how the 49 are categorised. It is possible that this is an error in annotation; it is not unusual for genes to be missed due to systematic issues of incomplete databases or lack of gene patterns in genomes

(Warren et al., 2010). Errors in assembly and breaks in contigs can also contribute to incorrect annotation (Salzberg, 2019). To avoid any issues with automatic gene builds and annotation, a manual gene curation could have been performed, whereby researchers manually inspect gene models to locate errors before analysis takes place (Pfeiffer & Oesterhelt, 2015).

**Table 3.** RAST output for genomic features of *S. uberis* Genome 14

| **Features** | ***S. uberis* Genome 14** |
|---|---|
| Genome size (bp) | 1,873,945 |
| GC content (%) | 36.4 |
| Number of contigs (with protein encoding genes) | 72 |
| Protein encoding genes | 1878 |
| Total RNA genes | 49 |

bp = base pairs, GC = guanine-cytosine

## 4.5 Antibiotic Resistance Gene Identification

Antibiotic resistance genes were identified using CARD. Selection criteria included perfect, strict, and loose hits to capture all potential homologs. The RGI output for the CARD analysis on genome 14 did not result in any perfect hits. Two strict hits were identified, along with 125 loose hits. The strict and loose hits of interest are displayed in Table 4. No other loose hits in CARD were pursued due to low % (< 65) identity of matching region, indicating they are unlikely to be significant hits.

A strict hit was returned for the *lnu*(C) gene at 97.56% identity. The *lnu*(C) gene confers resistance to the lincomycin antibiotic by adenylating the antibiotic with a nucleotidyltransferase protein, which inactivates the lincomycin (Achard et al., 2005). The *lnu*(C) gene was first identified in *S. agalactiae*, a well-established human pathogen, after displaying an atypical antibiotic resistance phenotype following clinical isolation (Achard et al., 2005). Its presence in bovine mastitis-associated *S. uberis* is novel, with only one record of it in the literature; this suggests it has been only recently attained (Vezina et al., 2021). Though uncommon, as there is recent evidence of this gene in bovine mastitis-associated *S. uberis* it was considered feasible that it may be present. Additionally, *lnu*(C) is transposon-mediated which means intercellular genome integration is possible (Achard et al., 2005; De Luca et al., 2018).

The other strict hit returned was for the *patB* gene at 68.29% identity. The protein patB confers resistance to fluroquinolone antibiotics by forming a protein heterodimer with patA, which creates an ATP binding cassette (ABC) superfamily efflux system that transports the antibiotic out of the cell (El Garch et al., 2010). The gene *patA* was identified as a loose hit at

66.13% identity. The genes *patA* and *patB* have their own roles in the metabolic and growth processes of the cell; however, when exposed to fluroquinolones they become overexpressed within the cell (Boncoeur et al., 2012; Marrer et al., 2006). Resistance conferred by patA/patB has been shown thus far in *Streptococcus pneumonaie* isolates (Marrer et al., 2006). A search of the literature did not result in any reported *S. uberis* isolates with *patA/patB*-mediated antibiotic resistance. With a lower % identity for both hits, and no precedent in literature, it is possible that CARD has incorrectly identified homologous ABC superfamily efflux system genes which have similar function to *patA* or *patB*. It is also possible that *pat*A/*pat*B are present as part of the core genome in *S. uberis*, but do not notably confer resistance to fluoroquinolones thus are not studied through this lens. Streptococci typically have low-level intrinsic resistance to quinolones, which has previously been thought due to the presence of the major facilitator superfamily antibiotic efflux pump protein PmrA; recent investigations have found this to not be correct (Brenwald et al., 2003; Garvey & Piddock, 2008).

Unfortunately, due to the interruptions in lab work, the ability to compare resistance phenotypes from the antibiotic susceptibility testing to the RGI output from CARD is not possible. This limits the discussion of how successful CARD was in identifying potential resistance genes, or further investigation and curation of lower-scored CARD hits that match any resistance phenotypes.

**Table 4.** CARD hit analysis for antibiotic resistance genes of interest for *S. uberis* Genome 14

| RGI criteria | ARO term | AMR gene family | Drug class | Resistance mechanism | % Identity of matching region | % Length of reference sequence |
|---|---|---|---|---|---|---|
| Strict | *lnu*(C) | lincosamide nucleotidyltrans-ferase (LNU) | Lincosamide antibiotics | Antibiotic inactivation | 97.56 | 114.02 |
| Strict | *patB* | ABC antibiotic efflux pump | Fluoroquinolone antibiotic | Antibiotic efflux | 68.29 | 101.02 |
| Loose | *patA* | ABC antibiotic efflux pump | Fluoroquinolone antibiotic | Antibiotic efflux | 66.13 | 100.89 |

## 4.6 Comparative genomics

For CARD strict hits, the genes *lnu*(C) and *patB* were recognised and investigated (Table 4). Despite being a loose hit, *patB* was also chosen for investigation as the protein produced exists as part of an ATP-binding cassette superfamily (ABC) efflux transporter system along with patA (El Garch et al., 2010).

### 4.6.1 *lnu*(C)

The *lnu*(C) gene has been found to express a nucleotidyltransferase protein that confers resistance to the antibiotic lincomycin by adenylation of the antibiotic molecule, resulting in inactivation (Achard et al., 2005). Using a confirmed *lnu*(C) CDS, a high similarity BLASTn search against genome 14 located a 100% percentage identity (ID) and query cover (QC) result on contig 3. This was considered to be the presumptive *lnu*(C) gene flagged by CARD. The same CDS was used to perform a high similarity BLASTn query against the entire database. This returned a significant alignment (ID 98.99%, 100% QC, E = 0.0) for *Streptococcus agalactiae* UCN36 *lnu*(C), which is the first characterisation of this gene in literature (Achard et al., 2005).

As *lnu*(C) is transposon-mediated (Achard et al., 2005; De Luca et al., 2018), investigation of any associated transposase CDS was performed. The aforementioned *S. uberis* isolate was used to locate a preceding incomplete transposase CDS. This was used to perform a high similarity BLASTn search against genome 14 (ID 100%, QC 100%, E = $2.0 \times 10^{-177}$) for a non-annotated region on contig 3 next to the presumptive *lnu*(C). This non-annotated region of genome 14 was used to perform a high similarity BLASTn query against the entire database, resulting in significant alignment for *S. agalactiae* strain UCN36 transposon MtnLNU transposase-like (insLNU) (ID 99.70%, QC 99%, E = $5.0 \times 10^{-173}$). This finding supported the presence of the transposon associated with *lnu*(C).

To provide further evidence for the presence of the transposase associated with *lnu*(C), the region from nucleotide 13,406 to 15,412 on contig 3 of genome 14 which contained the presumptive *lnu*(C) and transposase was submitted to IS Finder using a high similarity BLAST query. This resulted in a significant alignment with ISSag10, IS Family IS1595 and group ISPna2 originating in *S. agalactiae* (E = 0.0). This contrasts with the *S. uberis* with confirmed *lnu*(C) found in literature, which identified the IS family as IS3 (Vezina et al., 2021). However, ISSag10 is known to hold the *lnu*(C) gene (Achard & Leclercq, 2007; Siguier et al., 2009). It is possible that the search in IS finder misidentified the sequence as the RAST annotation failed to identify a start codon for the transposase, subsequently returning an incorrect sequence result. However, the literature suggests this finding is reasonable as the ISSag10 from the IS1595 family has been found to contain *lnu*(C).

Based on these findings, and the findings of Vezina et al. (2021), the presence of *lnu*(C) in and the associated transposase in *S. uberis* genome 14 is plausible and suggests some type of acquired resistance due to horizontal gene transfer from *S. agalactiae*. Given the prevalence of *S. uberis* as a major bovine mastitis pathogen on NZ dairy farms, interspecies acquired resistance to antibiotics via mobile elements is of great concern.

Though not utilised in high numbers in NZ, lincomycin in conjunction with neomycin is used as a second line intramammary therapy against staphylococcal mastitis in lactating cows (MPI, 2020; NZVA, 2018). Therefore, if *lnu*(C) is functioning and expressed in bovine-associated *S. uberis*, it is conceivable that second-line therapy of this type would no longer work against infection. Lincomycin resistance in bovine-associated *S. uberis* is not novel in itself, though has typically been attributed to the genes *lin*(B) and *lnu*(D) (Haenni et al., 2010; Petinaki et al., 2008; Petrovski et al., 2015). Though cross antibiotic resistance between lincosamide and macrolide antibiotics can occur due to similar modes of action in the cell, as *lnu*(C) is drug-specific against lincomycin this is likely not a concern (Petinaki & Papagiannitsis, 2018).

### 4.6.2 *patA*/*patB*

The genes *patA* and *patB* have been demonstrated to produce proteins that heterodimerise, forming a multidrug efflux transporter which confers fluroquinolone resistance (Boncoeur et al., 2012; Robertson et al., 2005). Though patA and patB have been shown to form homodimers, individually they do not appear to assist with antibiotic resistance (Boncoeur et al., 2012). A reference *pat*A CDS was located in *S. pneumoniae* strain R6 (accession number: AE007317.1). The CDS for *patA* was isolated and used in a high similarity BLASTn query against genome 14, which resulted in one significant alignment (ID 67.7%, QC 86%, E = $5.0 \times 10^{-142}$) on contig 2. The presumptive *patA* CDS from genome 14 was used to perform a high similarity BLASTn search against the entire data base which returned multiple significant alignment results for *S. uberis*.

Using the previous *S. pneumoniae* isolate, the confirmed *patB* CDS was used to perform a somewhat similar BLASTn query against genome 14. This resulted in the location of a significant alignment (ID 69.2%, QC 83%, E = $1.0 \times 10^{-169}$) on contig 2 next to the previously identified *patA*.

The presumptive *patA* and *patB* on genome 14 was located on contig 2 using Geneious. It was found that RAST had annotated these genes as *lmrC* and *lmrD,* respectively. This annotation is unlikely to be accurate as these genes are not found in streptococci, but rather *Lactococcus lactis* and *Streptomyces lincolnensis* (Lubelski et al., 2006; Xu et al., 2020). The genes *lmr*C/*lmrD* are ABC transporters that are thought to function as a pair which have previously been connected to efflux-mediated multidrug resistance activity; thus, they share function and are close homologues of *patA*/*patB* (Garvey & Piddock, 2008).

Fluoroquinolones act on bacteria by inhibiting action of topoisomerase proteins which halts DNA replication (Grobbel et al., 2007). Fluoroquinolone use in NZ dairy cattle is sparing due to its status as a ‘red light’ antibiotic (MPI, 2020; NZVA, 2018). However, fluoroquinolone sales for

cattle have increased by approximately 60% since 2016, implying that usage is growing (MPI, 2020). It should be noted that the *S. uberis* isolates provided for genomic analysis were collected previous to 2011. The fluoroquinolone antibiotics marbofloxacin and enrofloxacin are indicated for use in Gram-negative mastitis infections, therefore not necessarily relevant to the treatment of bovine-associated *S. uberis.* As previously acknowledged, many streptococci already have low-level intrinsic resistance to quinolones due to active efflux mechanisms, and point mutations in the quinolone resistance-determining regions of *gyrA* and *parC* (Sahasrabhojane et al., 2014). This study did not check for presence of *gyrA* and *parC*; this should be carried out during further investigations. As interspecies horizontal gene transfer of the quinolone resistance-determining regions in streptococci has been observed, remaining vigilant about the emergence of increased fluoroquinolone resistance in *S. uberis* is prudent (Ip et al., 2007).

These findings in conjunction with what is seen in literature lead to an inconclusive result as to the likelihood of the accuracy of the CARD hit for *patA*/*patB*. There does not appear to be any precedent for *patA*/*patB*-associated antibiotic resistance in *S. uberis* in literature. It is possible that these genes are present as part of the genome of *S. uberis* but do not take part in antibiotic resistance. Additionally, these genes could be another ABC transporter and close homologue of *patA*/*patB* or *lmr*C/*lmrD*. Further investigation into *patA*/*patB* could include conserved domain analysis, whereby protein similarity and shared function could be assessed by aligning the confirmed *patA*/*patB* with genome 14's presumptive *patA*/*patB* (Bradshaw et al., 2011).

### 4.6.3 Limitations

As this aspect of the research only encompassed a bioinformatics approach, there are limitations in what can be inferred from these findings. Studies of this nature often comprise an investigation into both phenotype and genotype resistance profiles, which would include antibiotic susceptibility testing followed by genomic analysis. Though genomic analysis can suggest a gene is present, without further experimentation it is impossible to know whether any genes found by CARD are actively expressed by the bacteria. In order to know if any of the genes are being expressed, transcriptomics techniques—such as reverse transcription polymerase chain reaction (RT-PCR) to amplify the messenger RNA for any proteins of interest—would have to be carried out (Milward et al., 2016). Gene knockout studies, whereby the phenotype of the organism is altered by systematically removing specific genes, could also be performed to understand if the genes are responsible for antibiotic resistance.

# 5.0 Future Directions

Due to restraints on time and budget, there were limitations on the scope of this study. In order to address the limitations noted, and expand on the knowledge gained from this research, the following areas are suggested for future study.

- **Repeating the antibiotic susceptibility testing, including MIC testing, with Mueller-Hinton agar and fresh antibiotics.** This would allow for more accurate comparisons with clinical standards for zone of inhibition breakpoints, which in turn would more accurately identify which isolates should be submitted to further MIC testing.
- **Phenotypic testing for *S. uberis* against a selection of fluoroquinolones and lincomycin.** Taking the *S. uberis* genome 14 isolate, if available, and performing antibiotic susceptibility testing (including MIC testing) to see if the resistance genes identified by CARD analysis do indeed confer resistance to fluoroquinolone and lincomycin antibiotics. This would give important information about the accuracy and precision of CARD hit analyses.
- **Perform a conserved domains analysis on the patA/patB proteins.** This would potentially identify proteins from the same ABC transporter family with similar function which are more likely to be in the genome of *S. uberis*, or give further evidence as to whether it is plausible at *patA*/*patB* are indeed present.
- **Perform an RT-PCR on the *S. uberis genome 14* isolate to confirm expression of *patA*/*patB* and *lnu*(C).** To discover if the *S. uberis* genome 14 isolate is actively producing patA/patB and *lnu*(C) proteins, an RT-PCR could be performed. This would help confirm whether the genes are active in the genome of this isolate. Additionally, if the conserved domain analysis reveals that other similar genes with shared function may be present, those genes should also be subject to RT-PCR analysis.
- **Carry out knockout studies in conjunction with phenotypic susceptibility testing for *patA*/*patB* and *lnu*(C) if they are found to be present, to see if the removal of these genes influences antibiotic susceptibility.** Even if the genes are present and actively expressed, it may not necessarily mean that their expression confers resistance to antibiotics, or if resistance occurs, that it is because of these genes. Knockout studies along with phenotypic testing might help researchers to confirm these specific genes confer resistance in this specific S. *uberis* isolate.

# 6.0 Conclusions

Antibiotic resistance surveillance is an important aspect of combatting AMR in the environment. In order to overcome the challenges involved in AMR, it is crucial to observe changes in patterns in antibiotic resistance, especially in known contagious and environmental pathogens. This research sought to understand the prevalence of ARB in bulk raw milk tanks from NZ dairy farms using antibiotic susceptibility testing. Additionally, genomic analysis of a known bovine mastitis pathogen was undertaken to identify genes that may confer antibiotic resistance to the bacterium.

During antibiotic susceptibility testing it was found that the presumptive *Staphylococcus* isolates displayed a high level of resistance to β-lactam antibiotics including the second-generation cephalosporin, cefuroxime. In the *Streptococcus*/*Enterococcus* isolates, 100% resistance was observed in response to penicillin G, but were otherwise highly susceptible to the other β-lactam antibiotics. The MRSA isolate showed a standard antibiotic resistance profile; however, the quality control *S. aureus* strain displayed some unexpected resistance against the majority of the β-lactam antibiotics. This could suggest that some of the antibiotics used did not have the expected efficacy, though issues with the control strain are also possible. Though vancomycin resistance is only able to be accurately assessed using MIC testing, a variety of inhibition zone sizes were recorded across all isolate groups. This indicates a need for further investigation for any test resulting in a small zone of inhibition.

For the genomic analysis of *S. uberis* genome 14, the ARG *lnu*(C), and *patA*/*patB* were identified by CARD. Further analysis showed that while *lnu*(C) is likely to have been accurately identified by CARD, evidence for the presence of *patA*/*patB* is inconclusive. Further testing, using both phenotypic and genotypic techniques, is necessary to understand if these genes are present, and if they confer antibiotic resistance.

This study has shown evidence that ARB may be present within New Zealand dairy cattle, including some novel observations of horizontally-transferred resistance determinants. It highlights the need for ongoing research in this area.

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